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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Paul C. Anderson *et al.*

Serial No.: 09/732,439

Filed: December 7, 2000

For: TRANSGENIC MAIZE WITH INCREASED PROLINE CONTENT (AS AMENDED)

Group Art Unit: 1638

Examiner: Cynthia E. Collins

Atty. Dkt. No.: DEKM:184USD1

CERTIFICATE OF MAILING
37 C.F.R. §1.8

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November 23, 2004
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Robert E. Hanson

BRIEF ON APPEAL

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BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief in response to the Office Action dated August 23, 2004. An Amendment Under 37 C.F.R. §1.116 is being concurrently filed. The date for filing this Brief is November 23, 2004, based on the date of the Office Action.

The fees for filing this Appeal Brief were filed with the Appeal Brief dated March 12, 2004 and thus it is believed that no fee is due. However, should any such fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the enclosed materials, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski Deposit Account No. 50-1212/DEKM:184USD1.

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I. REAL PARTY IN INTEREST

The real party in interest is Monsanto Company, the parent company of assignee DeKalb Genetics Corp.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-58 were originally filed. Claims 59-96 were added and claims 1-58 were cancelled in the Preliminary Amendment mailed on December 7, 2000. In response to a Restriction Requirement, Appellants elected Group I, comprising claims 59-63 and 72-73. Claims 64-71 and 74-96 were subsequently withdrawn from consideration by the Examiner as drawn to non-elected subject matter.

An Amendment Under 37 C.F.R. §1.116 was filed with the first Appeal Brief on March 12, 2004 and was entered by the Examiner. A second Amendment Under 37 C.F.R. §1.116 is being concurrently filed.

Claims 59-63 and 72-73 were pending and under consideration at the time of the Office Action dated August 23, 2004. These claims are still pending, under consideration and are the subject of this Appeal. A copy of the appealed claims as they stand without entry of the concurrently filed Amendment Under 37 C.F.R. §1.116 is attached as Appendix 1 and a copy of the claims as they stand with entry of this Amendment is attached as Appendix 2.

IV. STATUS OF AMENDMENTS

An Amendment Under 37 C.F.R. §1.116 was filed with an Appeal Brief on March 12, 2004 and was entered by the Examiner.

A second Amendment Under 37 C.F.R. §1.116 is being filed concurrently filed amending claim 63 to specify that the claimed seed includes the recombinant DNA segment of the transgenic plant of claim 61. The status of the Amendment is unknown. No other claim amendments have been made subsequent to the Office Action.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention relates to methods for the creation of transgenic monocot plants and seeds that display increased water stress resistance or tolerance. Specification at page 3, lines 20-24. More particularly, it concerns expression in monocots of an enzyme that catalyzes the synthesis of the osmoprotectant proline. Specification at page 3, lines 26-29, and page 4, line 8.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

(A) Are claims 59-63 and 72-73 properly rejected under 35 U.S.C. § 112, first paragraph, as lacking an adequate written description in the specification?

(B) Are claims 59-63 and 72-73 properly rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement in the specification?

(C) Are claims 61-63 properly rejected under 35 U.S.C. § 112, second paragraph, as being indefinite?

(D) Is claim 63 properly rejected under 35 U.S.C. § 101 as directed to non-statutory subject matter?

(E) Are claims 59-61, 63, 72 and 73 properly rejected under 35 U.S.C. § 102(e) as being anticipated by Verma *et al.* (U.S. Patent 5,639,950)?

(F) Are claims 59-63 and 72-73 properly rejected under 35 U.S.C. § 103(a), as being obvious over Verma *et al.* (U.S. Patent 5,639,950) in view of Rayapati *et al.* (*Plant Physiology*, 1989, vol. 91, pages 581-586) and Appellants allegedly admitted prior art?

VII. ARGUMENT

A. The Written Description Rejection Was Improperly Maintained

The Examiner has rejected claims 59-63 and 72-73 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to convey to one of skill in the art that Appellants were in possession of the claimed subject matter. In particular, the Examiner alleges that Appellants were not in possession of recombinant DNA molecules describing the genus of biosynthetic enzymes that catalyze the synthesis of the osmoprotectant proline.

In response, Appellants note that genes encoding enzymes that elevate the level of proline were known in the art at the time of filing. It is well settled that the specification need not disclose what is well-known to those skilled in the art and ***preferably omits*** what is well-known and already available to the public. *See Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986). For example, Verma *et al.* (U.S. Patent 5,344,923, **Exhibit A**), which the Examiner cites in the prior art rejection, discloses the isolation of a mothbean cDNA clone encoding a bifunctional enzyme, delta1-pyrroline-5-carboxylate synthetase, which is involved in the biosynthesis of proline in plants. Similarly, a soybean homologue of delta1-pyrroline-5-carboxylate synthetase was disclosed in 1992 by Hu *et al.* in *Proceedings National Academy of Science* (89:9354-9358; **Exhibit B**). Hu *et al.* also disclose

that the enzyme catalyzes the first two steps in proline biosynthesis in plants. Verbruggen *et al.*, teach that pyrroline-5-carboxylate reductase encodes the last step of the proline biosynthetic pathway and describes the cloning of the corresponding gene from *Arabidopsis* ("Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis* thaliana" *Plant Physiol.* Nov;103(3):771-81 (1993); **Exhibit K**). While the reference indicates a November 1993 publication date, it is indicative of the contemporaneous knowledge in the art. Additional examples of pyrroline-5-carboxylate reductases that were described include the human (Dougherty *et al.* "Cloning human pyrroline-5-carboxylate reductase cDNA by complementation in *Saccharomyces cerevisiae*" *J. Biol. Chem.*, 267 (2), 871-875 (1992); **Exhibit L**) and yeast genes (Brandriss *et al.* "Proline biosynthesis in Saccharomyces cerevisiae: analysis of the PRO3 gene, which encodes, which encodes delta 1-pyrroline-5-carboxylate reductase" *J. Bacteriol.* 174 (15), 5176 (1992); **Exhibit M**). Finally, Williamson and Slocum describe the cloning of a delta1-pyrroline-5-carboxylate reductase gene from pea (*Plant Physiol.*, 1992, 100, 1464-1470; **Exhibit N**).

As these sequences were known to those of skill in the art at the time of filing, Appellants cannot be said to lack written description for these sequences. A person of ordinary skill in the art would have known of useful gene sequences involved in the synthesis of proline at the time of Appellants' invention. That the availability of such gene sequences was common knowledge obviates the rejection under 35 U.S.C. §112, first paragraph. Written description must be reviewed from the perspective of one of skill in the art. *Wang Labs., Inc. v. Toshiba Corp.*, 993 F.2d 858, 863 (Fed. Cir. 1993). There is therefore an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy written description. USPTO Written Description Guidelines, Fed. Reg. Vol. 66, p. 1105. Applicants

need not specifically recite what is well known. The evidence presented therefore demonstrates full compliance with the written description requirement and reversal of the rejection is thus respectfully requested.

B. The Enablement Rejection Was Improperly Maintained

The Examiner finally rejected claims 59-63 and 72-73 under 35 U.S.C. §112, first paragraph, as not being enabled by the specification. In particular, the Examiner takes issue with an alleged failure to disclose the identification or isolation of a particular gene and/or plant comprising recombinant DNA encoding an enzyme involved in proline synthesis.

In response, Appellants note that the claims are fully enabled by the specification. The currently claimed invention forms a part of a larger invention comprising use of compatible osmoprotectants in monocots in order to achieve resistance to a reduction in water availability.

As described in the specification, Appellants' method for transformation of monocots with osmoprotectant genes, combined with known examples of enzymes synthesizing proline in particular, enables a person of ordinary skill in the art to produce transgenic monocots that express proline and exhibit water stress tolerance. For example, Appellants have illustrated this in the context of transgenic plants that express the osmoprotectant mannitol (see US Patent 5,780,709, **Exhibit D**) and glycine betaine producing enzymes (see U.S. Patent 6,281,411, **Exhibit C**). Proline, like mannitol and glycine betaine, is a known endogenous osmoprotectant that has long been identified as playing a role in plants under water deficit. What was not known, and the Appellants demonstrate, is that such genes could be expressed heterologously in monocots to yield a drought-tolerant phenotype.

In the case of proline, Barnett *et al.* (*Plant Phys.*, 41:1222, 1966; **Exhibit E**) describes that under water deficit, significant increases in certain amino acid pools such as proline are

observed. Wyn Jones and Storey (*Physiology and Biochemistry of Drought Resistance in Plants*, Chapter 9, p. 171-204, Academia Press, Australia, 1981; **Exhibit F**) notes that increased proline accumulation is observed in barley subjected to water or salt stress. McCue and Hanson (*TIBTECH*, 8:358-362, 1990; **Exhibit G**) specifically mention the amino acid proline as an osmoprotectant found in diverse organisms. Van Rensberg *et al.* (*J. Plant. Physiology*, 141:1880194, 1993; **Exhibit H**) discuss their observations of increased proline accumulation in drought-resistant tobacco cultivars, where a substantial amount of proline was found to accumulate in the drought-resistant cultivars compared to the drought-sensitive cultivars.

Appellants teach that heterologous gene expression to increase mannitol and glycine betaine imparts water stress tolerance to transgenic monocot plants (see US Patent 5,780,709 **Exhibit D** and US Patent 6,281,411 **Exhibit C,** respectively). This and success in using the mtlD gene to impart drought tolerance to a monocot are therefore demonstrative of success in overexpressing proline to obtain water stress tolerance. Together, the knowledge of proline accumulation in plants in response to drought stress, as well as the knowledge of sequences involved in the synthesis of proline demonstrate that the claims were enabled for expressing a proline biosynthesis gene and increased water stress tolerance. In fact, the Examiner's own prior art rejection states that expression of a delta1-pyrroline-5-carboxylate synthetase would inherently result in water stress tolerance. Given the transformation methods and expression vectors illustrated in Appellants' working examples to be fully enabling for monocot transformation and heterologous expression with osmoprotectant genes, it is respectfully submitted that the claims must be held enabled in view of the assertion. While legally flawed from an inherency rejection standpoint because the cited references are not enabling for transgene expression in maize and the insufficiency of Verma as of its effective date, the

rejection affirmatively acknowledges the enablement of the claims given Appellants' enablement of transgene expression in monocots.

Reversal of the Section 112 rejection for lack of enablement is thus respectfully requested.

C. The Indefiniteness Rejections Were Not Properly Maintained

Independent claim 61 and claims 62-63 dependent thereon stand rejected under 35 U.S.C. §112, second paragraph, as being indefinite for the use of the term "increased" in claim 61. It is stated that the term is relative, lacking comparative basis, and that the definition in the specification is not limiting.

Claim 61 reads as follows:

> 61. A fertile transgenic *Zea mays* plant comprising a recombinant DNA segment comprising a promoter operably linked to a first DNA segment encoding an enzyme which catalyzes the synthesis of the osmoprotectant proline, wherein the first DNA segment is expressed so that the level of the enzyme is ***increased*** in the transgenic *Zea mays* plant, and wherein the recombinant DNA segment is heritable.
>
> (emphasis added)

As can be seen from the text of the claim, the meaning of "increased" is clear to one of skill in the art, particularly when taken in combination with the teaching in the specification. A plain reading of the claim indicates that the enzyme is increased relative to a *Zea mays* plant that lacks the recombinant DNA segment. No other logical reading can be made of the claim given the text. The Examiner has failed to point to any other reasonable meaning that could be given.

There is no indefiniteness in using a relative term that has a comparative basis. All that is required under the second paragraph of §112 is that one of skill in the art understand the metes and bounds of the claim when read in context and in view of the specification. Claim terms must

be read together with the claim as a whole and in view of the understanding of those of skill in the art. Properly viewed, the claim is fully definite.

In light of the foregoing, reversal of the rejection under 35 U.S.C. § 112, second paragraph, is respectfully requested.

D. The Rejection of Claim 63 Under 35 U.S.C. §101 is Moot

The Examiner rejected claim 63 under 35 U.S.C. §101 as directed to non-statutory subject matter for not specifying that the claimed seed is transgenic, and therefore, not a product of nature. In response, it is noted that an Amendment Under 37 C.F.R. §1.116 has been concurrently filed specifying that the seed includes the recombinant DNA segment recited in claim 61. The rejection is therefore now believed to be moot.

E. The Anticipation Rejection Under 35 U.S.C. §102(e) Was Not Properly Maintained

The Examiner has rejected claims 59-61, 63, 72 and 73 under 35 U.S.C. §102(e) as being anticipated by Verma *et al.* (U.S. Pat. No. 5,639,950, issued June 17, 1997, filed June 29, 1994 as a CIP of an application with an effective filing date of September 29, 1992 (hereinafter Verma II, **Exhibit I**)). The September 29, 1992 priority document of Verma II (hereinafter Verma I) issued as U.S. Patent No. 5,344,923 (**Exhibit A**).

The rejection is insufficient on its face. Appellants noted in their response to the first Office Action that the current application claims priority to August 25, 1993 as a divisional application of U.S. Application Serial No. 08/599,714, filed January 19, 1996 (now U.S. Patent No. 6,281,411), which application was a continuation-in-part application of currently pending U.S. Application Serial No. 08/113,561, filed August 25, 1993. This grandparent application disclosed that transgenic monocot plants with drought resistance, including maize, can be

prepared by expressing genes encoding a variety of osmotically active metabolites including proline.

The Verma II patent is effective only as of its June 1994 filing date for disclosure of "corn" and thus cannot anticipate the current claims. The Examiner nonetheless relies on Verma I based on the allegation that this disclosure is sufficient because there is allegedly no difference in the teachings with respect to transgenic plants. However, the shortcomings of Verma II do not supplement the shortcoming of Verma I. What is relevant is that Verma I is insufficient relative to the claimed invention.

A full text search of the Verma I patent (U.S. Pat. No. 5,344,923) on the USPTO patent database reveals that this patent does ***not include*** the terms ***"maize," "Zea mays," "corn" or "monocot."*** The Examiner's rejection is therefore fundamentally without merit. It cannot be alleged that the patent anticipates transgenic monocots and maize expressing a particular transgene when it does ***not even disclose monocot or maize plants*** at all, let alone an enabling transformation method for such plants. To anticipate, ***all elements*** of the claims must be disclosed in a single reference. This has not been demonstrated and therefore the rejection fails.

More particularly, Appellants' claims are drawn to a transgenic monocot plant which is substantially tolerant or resistant to a reduction in water availability, where the transgenic plant comprises a transgene encoding an enzyme catalyzing the synthesis of proline. Verma I merely discloses the sequence of delta1-pyrroline-5-carboxylate synthetase, making the sequence available to those of ordinary skill in the art who might discover a use for it, *e.g.*, as Appellants have discovered a use in producing transgenic monocots expressing proline for drought resistance.

With regard to the Examiner's statement that plants disclosed by Verma I and/or II would "inherently be substantially tolerant or resistant to a reduction of water availability" it is first noted that this statement directly contradicts the enablement rejection. The Examiner cannot have it both ways. In view of the comment by the Examiner admitting that a monocot transformed with the delta1-pyrroline-5-carboxylate synthetase gene would inherently have a resistance to water availability, it is respectfully submitted that the Examiner has acknowledged the enablement of the claims.

It is further noted that the Examiner's conclusion regarding inherency is flawed. To form the basis of an inherency rejection, the missing item must necessarily flow from the disclosure. *Continental Can Co. USA v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991) ("To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill."). Here, the Examiner has failed to provide any objective basis to support the enablement of the Verma I or Verma II reference. For example, the Examiner has failed to show that Verma I and/or II disclose a method for transforming monocots and teach transformation vectors that could be used to achieve gene expression in monocots. Verma II does not even disclose monocots generally. The Examiner's unsupported allegations do not meet the relevant legal standards or the standards of the APA for maintaining a rejection.

The Examiner is not allowed to bootstrap deficient references to support an anticipation rejection through unsupported allegations of inherency. The law of inherency has been developed precisely so that the insufficiencies of a reference may not be glossed over by bare

assertions of inherency. Given the failure of Verma I to disclose transgenic maize or monocots at all or a showing that Verma II is even prior art as of its priority date, there is no basis to allege that the claimed transgenic monocot plants expressing an enzyme catalyzing proline biosynthesis necessarily flow from the disclosure.

In view of the foregoing, neither Verma I nor Verma II constitute a 35 U.S.C. §102(e) bar to the claims. Reversal of the rejection is thus respectfully requested.

F. The Obviousness Rejection Was Not Properly Maintained

The Examiner rejects claims 59-63, and 72-73 under 35 U.S.C. §103(a) as being unpatentable over Verma II (described above) in view of Rayapati *et al.* (*Plant Physiology*, 1989, 91:581-586; **Exhibit J**) and in light of Appellant's allegedly admitted prior art. The teachings of Verma II are discussed above. Rayapati *et al.* is cited for its finding that delta1-pyrroline-5-carboxylate synthetase is located in the chloroplast of peas.

In response, Appellants note that all elements of the claims have not been shown in the art and thus the rejection is insufficient on its face. First, the Examiner has failed to show monocot plants in the prior art. Instead, the Examiner makes the conclusory statement that she "maintains that Verma et al. teach corn, wheat, barley, and rye monocot plants comprising a recombinant Δ^1-pyrroline-5-carboxylate synthetase" that catalyzes proline synthesis. This assertion is unsupported and is baseless given that Verma I does ***not*** even reference ***maize or monocot plants*** as of its effective prior date.

The Examiner has therefore failed to make a *prima facie* case of obviousness for the current claims, which are directed to transformed monocots having DNA which encodes an enzyme catalyzing the synthesis of the osmoprotectant proline. Verma ***II*** has not even been

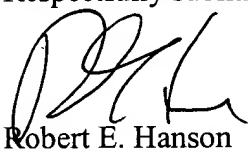
demonstrated by the Examiner to be properly used as prior art and does not even disclose an actual transgenic monocot plant. The defects of Rayapati *et al.* are not cured even based on this reference with an effective filing date after Appellants. The combined references do not teach fertile, transformed corn. The references have not been shown to provide a motivation to arrive at the claimed invention, let alone a reasonable expectation of success in doing so.

In conclusion, Appellants respectfully submit that there is no support for the rejection of claims 59-63 and 72-73 under 35 U.S.C. §103. Reversal of the rejection is thus respectfully requested.

VIII. CONCLUSION

It is respectfully submitted, in light of the above, that none of the claims are properly rejected. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,



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Date: November 23, 2004

APPENDIX 1: LISTING OF APPEALED CLAIMS PRIOR TO ENTRY OF THE CONCURRENTLY-FILED AMENDMENT UNDER 37 C.F.R. §1.116

59. A transformed monocot plant, which plant is substantially tolerant or resistant to a reduction in water availability, the cells of which comprise a recombinant DNA segment comprising a preselected DNA segment encoding an enzyme which catalyzes the synthesis of the osmoprotectant proline, wherein the enzyme is expressed in an amount effective to confer tolerance or resistance to the transformed plant to a reduction in water availability.

60. The transformed plant of claim 59 wherein the transformed plant has an improved osmotic potential when the total water potential of the transformed plant approaches zero.

61. A fertile transgenic *Zea mays* plant comprising a recombinant DNA segment comprising a promoter operably linked to a first DNA segment encoding an enzyme which catalyzes the synthesis of the osmoprotectant proline, wherein the first DNA segment is expressed so that the level of the enzyme is increased in the transgenic *Zea mays* plant, and wherein the recombinant DNA segment is heritable.

62. The fertile transgenic *Zea mays* plant of claim 61, wherein the recombinant DNA segment further comprises a second DNA segment encoding an amino terminal chloroplast transit peptide operably linked to the first DNA segment.

63. A seed produced by the transgenic plant of claim 61.

72. A transformed monocot plant regenerated from the transformed plant cells obtained by the method of claim 64.

73. A transgenic seed of the transformed plant of claim 72 which comprises the expression cassette.

APPENDIX 2: LISTING OF APPEALED CLAIMS AFTER ENTRY OF THE CONCURRENTLY-FILED AMENDMENT UNDER 37 C.F.R. §1.116

59. A transformed monocot plant, which plant is substantially tolerant or resistant to a reduction in water availability, the cells of which comprise a recombinant DNA segment comprising a preselected DNA segment encoding an enzyme which catalyzes the synthesis of the osmoprotectant proline, wherein the enzyme is expressed in an amount effective to confer tolerance or resistance to the transformed plant to a reduction in water availability.

60. The transformed plant of claim 59 wherein the transformed plant has an improved osmotic potential when the total water potential of the transformed plant approaches zero.

61. A fertile transgenic *Zea mays* plant comprising a recombinant DNA segment comprising a promoter operably linked to a first DNA segment encoding an enzyme which catalyzes the synthesis of the osmoprotectant proline, wherein the first DNA segment is expressed so that the level of the enzyme is increased in the transgenic *Zea mays* plant, and wherein the recombinant DNA segment is heritable.

62. The fertile transgenic *Zea mays* plant of claim 61, wherein the recombinant DNA segment further comprises a second DNA segment encoding an amino terminal chloroplast transit peptide operably linked to the first DNA segment.

63. A seed produced by the transgenic plant of claim 61 comprising said recombinant DNA segment.

72. A transformed monocot plant regenerated from the transformed plant cells obtained by the method of claim 64.

73. A transgenic seed of the transformed plant of claim 72 which comprises the expression cassette.

APPENDIX 3: EVIDENCE APPENDIX

Exhibit A — U.S. Patent No. 5,344,923, cited in Action dated 8/23/04.

Exhibit B — Hu *et al.*, (*PNAS* 89:9354-9358) , cited in Action dated 8/23/04.

Exhibit C — U.S. Patent No. 6,281,411, cited in Action dated 8/23/04.

Exhibit D — U.S. Patent No. 5,780,709, cited in Action dated 8/23/04.

Exhibit E — Barnett *et al.* (*Plant Phys.*, 41:1222, 1966) , cited in Action dated 8/23/04.

Exhibit F — Wyn Jones and Storey (*Physiology and Biochemistry of Drought Resistance in Plants*, Ch. 9, p. 171-204, Academia Press, Australia, 1981), cited in Action dated 8/23/04.

Exhibit G — McCue and Hanson (*TIBTECH*, 8:358-362, 1990), cited in Action dated 8/23/04.

Exhibit H — Van Rensberg *et al.* (*J. Plant. Physiology*, 141:1880194, 1993), cited in Action dated 8/23/04.

Exhibit I — U.S. Patent No. 5,639,950, cited in Action dated 8/23/04.

Exhibit J — Rayapati *et al.* (*Plant Physiology*, 1989, 91:581-586), cited in Action dated 8/23/04.

Exhibit K — Verbruggen *et al.*, (*Plant Physiol.*, 1993, Nov; 103(3):771-81), cited in Action dated 8/23/04.

Exhibit L — Dougherty *et al.* (*J. Biol. Chem.*, 1992, 267 (2), 871-875) , cited in Action dated 8/23/04

Exhibit M — Brandriss *et al.* (*J. Bacteriol.*, 1992, 174 (15), 5176) , cited in Action dated 8/23/04

Exhibit N — Williamson and Slocum (*Plant Physiol.*, 1992, 100, 1464-1470), cited in this Brief concurrent with Appeal.

Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9354–9358, October 1992
Plant Biology

A bifunctional enzyme (Δ^1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants

(osmoregulation/nitrogen assimilation/functional complementation/root nodules)

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Communicated by Jozef S. Schell, July 1, 1992

ABSTRACT **Many plants synthesize and accumulate proline in response to osmotic stress. Despite the importance of this pathway, however, the exact metabolic route and enzymes involved in the synthesis of proline in plants have not been unequivocally identified. We report here the isolation of a mothbean (*Vigna aconitifolia*) cDNA clone encoding a bifunctional enzyme, Δ^1-pyrroline-5-carboxylate synthetase (P5CS), with both γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase activities that catalyzes the first two steps in proline biosynthesis. The two enzymatic domains of P5CS correspond to the ProB and ProA proteins of *Escherichia coli* and contain a leucine zipper in each domain, which may facilitate inter- or intramolecular interaction of this protein. The *Vigna* P5CS enzyme activity is feedback regulated by proline but is less sensitive to end-product inhibition than is the *E. coli* γ-glutamyl kinase. The P5CS gene is expressed at high levels in *Vigna* leaves and is inducible in roots subjected to salt stress, suggesting that P5CS plays a key role in proline biosynthesis, leading to osmoregulation in plants.**

Drought and high salinity are the most important environmental factors that cause osmotic stress and impact negatively on plant growth and crop productivity (1). To counter these stresses, many plants increase the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes such as proline and glycine betaine (2–4). The role of proline biosynthesis in osmoregulation in bacteria is well established (5); however, it is not known whether the accumulation of proline in plant tissues confers some adaptive advantage to the plant under osmotic stress or whether it is a consequence of stress-induced changes in metabolism (6). A complete understanding of stress-induced changes in the proline biosynthetic pathway and the regulation of genes involved in proline metabolism may reveal how plants adapt to environmental stresses. This could open the way for increasing tolerance to drought and salinity stresses by producing transgenic plants capable of synthesizing elevated levels of proline.

The proline biosynthesis route in plants (7, 8) is thought to resemble the pathway in bacteria (9). In *Escherichia coli*, the reaction starts with the phosphorylation of glutamate by γ-glutamyl kinase (γ-GK; encoded by the *proB* gene), to form γ-glutamyl phosphate, which is reduced to glutamic-γ-semialdehyde (GSA) by GSA dehydrogenase (encoded by the *proA* gene). GSA spontaneously cyclizes to Δ^1-pyrroline-5-carboxylate (P5C), which is reduced by P5C reductase (P5CR; encoded by the *proC* gene) to proline. The *E. coli proB*, *proA* (10), and *proC* (11) loci have been cloned and sequenced.

Whereas proline can be synthesized from either glutamate or ornithine in plants (7, 8) and animals (12–14), stable isotope and radioisotope labeling experiments (15–17) indicate that glutamate, rather than ornithine, is the primary precursor for proline biosynthesis in osmotically stressed plant cells. With the exception of P5CR, which has been recently characterized (18–20),‡ however, little is known about the other enzymes in this pathway. Although there is circumstantial evidence implicating a γ-GK in plants (7, 8), the existence of this activity has hitherto not been unequivocally demonstrated despite concerted efforts by several research groups over the past 25 years. Given the well-documented correlation between proline accumulation and adaptation to water deficits caused by drought and salinity stresses in plants, the complete characterization of the enzymes in the proline biosynthetic pathway and their biochemical and genetic regulation is vitally important.

We have recently isolated cDNA clones for soybean P5CR by direct complementation of an *E. coli proC* proline auxotroph with a soybean nodule cDNA expression library (20). The rationale of using a nodule library was based on the finding that high levels of proline are synthesized in soybean root nodules (21, 22). Using this complementation strategy (23), we have isolated from a mothbean (*Vigna aconitifolia*) nodule cDNA expression library cDNA clones coding for a bifunctional enzyme, P5C synthetase (P5CS), which exhibited both γ-GK and GSA dehydrogenase activities. The *Vigna* P5CS enzymatic activity was found to be feedback inhibited by proline. The level of P5CS transcript was shown to be enhanced in roots treated with salt, indicating that the expression of this gene is osmoregulated.§

MATERIALS AND METHODS

Bacterial Growth Conditions. *E. coli* mutant cells were grown at 37°C in LB medium, or in minimal A medium (23) supplemented with 0.2 mM required amino acids and 0.05 mM thiamine; ampicillin was added at 100 mg/liter. The osmotic strength of the medium was increased by the addition of NaCl as indicated.

Plasmids and Bacterial Strains. Plasmids carrying wild-type *proB* (pDU1) and mutant *proB74* (pDU101) alleles were obtained from Abhaya Dandekar (University of California, Davis). *E. coli* strains X340 (*proB28*, *metB1*, *relA1*, λ⁻, *spoT1*), W4032 (*proA3*, *metB1*, *relA1*, *lac-3*, *tsx-76*), CSH26 [see ref. 24; *ara*, Δ(*lac pro*), *thi*], and X342 (*proC29*, λ⁻,

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Abbreviations: γ-GK, γ-glutamyl kinase; GSA, glutamic-γ-semialdehyde; P5C, Δ^1-pyrroline-5-carboxylate; P5CR, P5C reductase; P5CS, P5C synthetase.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M92276).

§Verbruggen, N., Villarroel, R. & van Montagu, M. Third International Congress, International Society for Plant Molecular Biology, October 6–11, 1991, Tucson, AZ (abstr.).

relA1, *sptT1*, *metB1*) were kindly provided by Barbara Bachmann (*E. coli* Genetics Stock Center, Yale University).

Construction of cDNA Expression Library. RNA was prepared from 21-day-old mothbean (*Vigna aconitifolia* L.) nodules by the method of Verwoerd *et al.* (25), and poly(A)$^+$ RNA was isolated by chromatography on an oligo(dT)-cellulose column. Double-stranded cDNA was synthesized, ligated to *Bst*XI linkers, and size-fractionated on an agarose gel. Molecules >500 base pairs were electroeluted from the gel, cloned into the *Bst*XI-linearized pcDNAII plasmid vector (Invitrogen, San Diego) and *E. coli* DHIαF' cells were transformed. cDNA molecules inserted within the polylinkers of pcDNAII in the correct orientation and reading frame can be expressed under the control of the *lacZ* promoter.

Functional Complementation of *E. coli* Auxotrophs. *E. coli* proline auxotrophs were transformed by electroporation using Electro Cell Manipulator 600 apparatus (BTX, San Diego) according to the manufacturer's instructions. Briefly, cells were grown to late logarithmic phase in LB broth, pelleted by centrifugation, washed twice in ice-cold 10% (vol/vol) glycerol, resuspended in cold 10% glycerol, and stored in 200-μl aliquots at −80°C. For electroporation, 20 ng of the pooled library DNA purified by double CsCl centrifugation was added to 200 μl of competent cells in a 2-mm cuvette and subjected to a 5-msec pulse at 2.5 kV/cm. The cells were immediately added to 10 ml of LB broth and incubated at 37°C with shaking for 1 h. The cells were then pelleted by centrifugation, washed with minimal A medium, and plated onto minimal A plates containing ampicillin (100 μg/ml), isopropyl β-D-thiogalactopyranoside (1 mM), and the amino acid supplements other than proline required by the respective mutants. To determine the transformation efficiencies, aliquots of electroporated cells were plated onto the same media supplemented with proline (1 mM). Plates were incubated at 37°C for 2 days. Plasmid DNA, prepared from single colonies grown in minimal medium containing ampicillin, was used to retransform the mutants to confirm the complementation (see ref. 23).

DNA Sequencing and Analysis. DNA sequencing was performed on CsCl-purified, double-stranded plasmid DNA by the dideoxynucleotide chain-termination method using Sequenase II (United States Biochemicals) according to the manufacturer's instructions. Deletions of the plasmid template were generated by using Exonuclease III and mung bean nuclease. In addition, some sequencing reactions were performed with oligonucleotide primers synthesized on the basis of previously derived cDNA sequences. Sequence comparisons and identification of various motifs were done with Genetics Computer Group programs (Madison, WI).

P5CS Enzyme Assay and Determination of End-Product Feedback Inhibition. *E. coli* CSH26 cells and CSH26 harboring different plasmids were grown aerobically at 37°C for ≈16 h. The cells were harvested by centrifugation at 4000 × *g* for 10 min, and the cell paste was suspended in 4 ml of buffer A (50 mM Tris·HCl/1 mM dithiothreitol) at 4°C. After sonication (Benson Sonifier, type 450) at 50 W, the slurry was centrifuged at 4000 × *g* for 10 min and the supernatant was fractionated by ammonium sulfate precipitation. For *E. coli* CSH26 containing pVAB2, the fraction precipitating between 0% and 30% saturation of ammonium sulfate was used for enzyme assay; while for *E. coli* CSH26 and CSH26 harboring the *proB74* or wild-type *proB* alleles, a fraction of 40–60% saturation was used. Ammonium sulfate precipitated fraction was dissolved in buffer A and dialyzed against the same buffer. γ-GK activity was assayed by the method described by Hayzer and Leisinger (26). The amount of γ-glutamylhydroxamate made from L-glutamate, NH_2OH, and ATP was determined from the A_{536} after a 30-min incubation at 37°C. To determine whether proline biosynthesis is subject to end-product inhibition in plants, the γ-GK activities of the different ammonium sulfate fractions described above were measured as a function of proline concentration (0.01–100 mM) in the assay mixture.

Northern Blot Analysis. Poly(A)$^+$ RNA from mothbean nodules, leaves, and roots, as well as roots from plants watered with 200 mM NaCl, were electrophoresed in a 1.2% agarose/2.2 M formaldehyde gel and transferred by vacuum blotting to a GeneScreen membrane (DuPont/NEN). The pVAB2 cDNA insert was labeled with [^{32}P]dCTP to a specific activity of 8 × 10^8 dpm/μg using a random-primer labeling system (Amersham). Hybridization was performed as described (20), and filters were exposed to x-ray film at −70°C.

RESULTS AND DISCUSSION

Isolation of cDNA Clones Encoding *Vigna* P5CS. We have previously obtained a cDNA clone for soybean P5CR by direct complementation of an *E. coli proC* proline auxotroph with a soybean nodule cDNA expression library (20). To clone other plant enzymes of the proline biosynthetic pathway, we similarly transformed *E. coli proB*, *proA*, and *proBA* auxotrophic mutants by electroporation with a *V. aconitifolia* nodule cDNA expression library. A transformation efficiency of 10^9–10^{10} transformants per μg of DNA was routinely obtained, which is ≈2 orders of magnitude higher than that obtained by classical transformation methods (23); thus, electroporation significantly enhances the recovery of complemented mutants. One group of clones was isolated by selecting for growth of transformants on minimal medium without proline. We have determined that these clones encode a bifunctional enzyme, P5CS, which has both γ-GK and GSA dehydrogenase activities. This determination is based on the following evidence: the P5CS clones efficiently complemented *E. coli proB*, *proA*, and *proBA*, but not *proC*, mutants; the predicted amino acid sequence of the enzyme revealed the presence of two domains with significant homology to the *E. coli* ProB and ProA proteins; and the recombinant P5CS protein showed GSA-dependent γ-GK enzyme activity.

P5C can also be synthesized from ornithine via ornithine aminotransferase in plants, but the *E. coli* CSH26 cells are unable to grow on ornithine. These cells electroporated with library DNA were selected for proline prototrophy on minimal medium supplemented with ornithine (1 mM). With this approach, we obtained complementing clones encoding ornithine aminotransferase (unpublished data). These data demonstrate that proline is synthesized in plants from both glutamate and ornithine. The contribution of the respective pathway to proline production remains to be determined.

***Vigna* P5CS Contains Domains Homologous to *E. coli* ProB and ProA Proteins.** The complete nucleotide sequence of a P5CS cDNA clone, pVAB2, is shown in Fig. 1. The 2417-base-pair sequence contains a single major open reading frame encoding a polypeptide of 73.2 kDa, assuming that translation is initiated at the first ATG codon from the 5' end of the coding strand. Sequence comparison with the *E. coli* ProB and ProA proteins (Fig. 2) indicated that *V. aconitifolia* P5CS polypeptide has two distinct domains. The amino-terminal domain of the P5CS protein showed 33.3% identity and 55.3% overall similarity to the *E. coli* ProB protein. A domain with 35.7% identity and 57.9% similarity to the ProA protein is located at the carboxyl end. An unexpectedly high level (42.4%) of sequence similarity was found between the *E. coli* ProB and ProA proteins, suggesting that the *proB* and *proA* genes may have arisen by duplication of an ancestral gene; the encoded proteins may have later acquired domains conferring the respective kinase and reductase activities of the present-day enzymes. The corresponding plant genes have evidently fused to create the bifunctional enzyme identified in *V. aconitifolia*. A similar event may have occurred

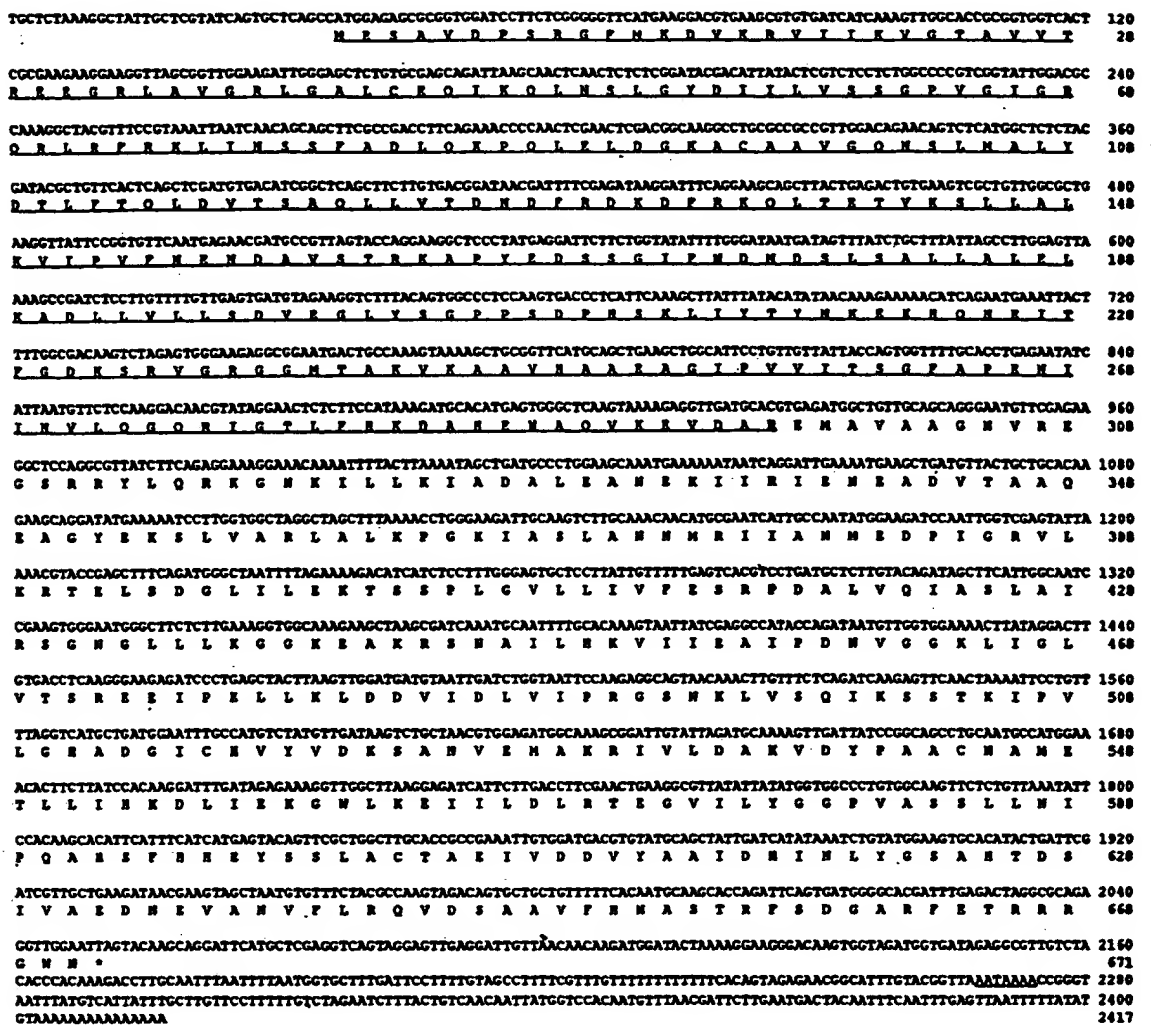

FIG. 1. Nucleotide sequence of the pVAB2 cDNA and primary sequence of the encoded *V. aconitifolia* P5CS. The polypeptide region homologous to the *E. coli* γ-GK (ProB) domain is underlined. The carboxyl half of P5CS corresponds to GSA dehydrogenase (ProA). A polyadenylylation signal at the 3′ end is underlined.

in animal systems since a P5CS activity has been detected in mammalian cells, although it is not known whether this is due to a single or to two separate enzymes (12–14).

Interestingly, a leucine zipper sequence (see Fig. 2) is present in each of the enzymatic domains of *Vigna* P5CS. The leucine zippers mediate protein:protein dimerization in structural proteins of both eukaryotes and prokaryotes as well as in transcriptional regulatory proteins (27, 28). The leucine zippers in P5CS may function intramolecularly to maintain the proper tertiary structure of the two domains of this enzyme, and homodimer or heterodimer formation may occur through the leucine zippers to allow close association between the originally separate enzymes. It is also possible that these zippers are a relic of the time when the γ-GK and GSA dehydrogenase enzymes were separate and had to be brought together to form a functional complex, as in *E. coli* (see ref. 9). Thus, these leucine zippers may facilitate inter- and intramolecular interactions. In addition, P5CS contains a potential phosphorylation site (Fig. 2). Site-directed mutagenesis may elucidate the significance of these domains and their role in the evolution of this bifunctional enzyme. Existence of two catalytic domains on the *Vigna* P5CS enzyme may facilitate sequential reactions of formation of glutamyl phosphate, which is unstable, and its rapid conversion into GSA. In *E. coli*, both γ-GK and GSA dehydrogenase function as hexameric enzymes (9) but the native size of P5CS is not yet determined.

P5CS Has GSA Dehydrogenase-Dependent γ-GK Activity That Is Feedback Inhibited by Proline. To confirm that pVAB2 encoded P5CS activity, ammonium sulfate-fractionated extracts of *E. coli* CSH26 and CSH26 harboring pVAB2 were assayed for GSA dehydrogenase-dependent γ-GK enzyme activity (26). As shown in Fig. 3, no γ-GK activity was detected in *E. coli* strain CSH26, whereas CSH26 containing pVAB2 exhibited a high level of γ-GK activity. Because proline biosynthesis is regulated by end-product inhibition of γ-GK in bacteria (26), we determined whether the plant enzyme is similarly subjected to feedback control. Fig. 3 shows that the *Vigna* enzyme is 50% inhibited by 6 mM proline. The wild-type *E. coli* γ-GK is 30 times more sensitive—i.e., shows 50% inhibition at 0.2 mM proline, while a mutant form of the enzyme (encoded by the *proB74* allele; refs. 30 and 31) is ≈200-fold less sensitive to end-product inhibition (Fig. 3; see also ref. 32). Thus, the sensitivity of the recombinant *Vigna* enzyme to feedback inhibition is much less than the wild-type *E. coli* enzyme but more

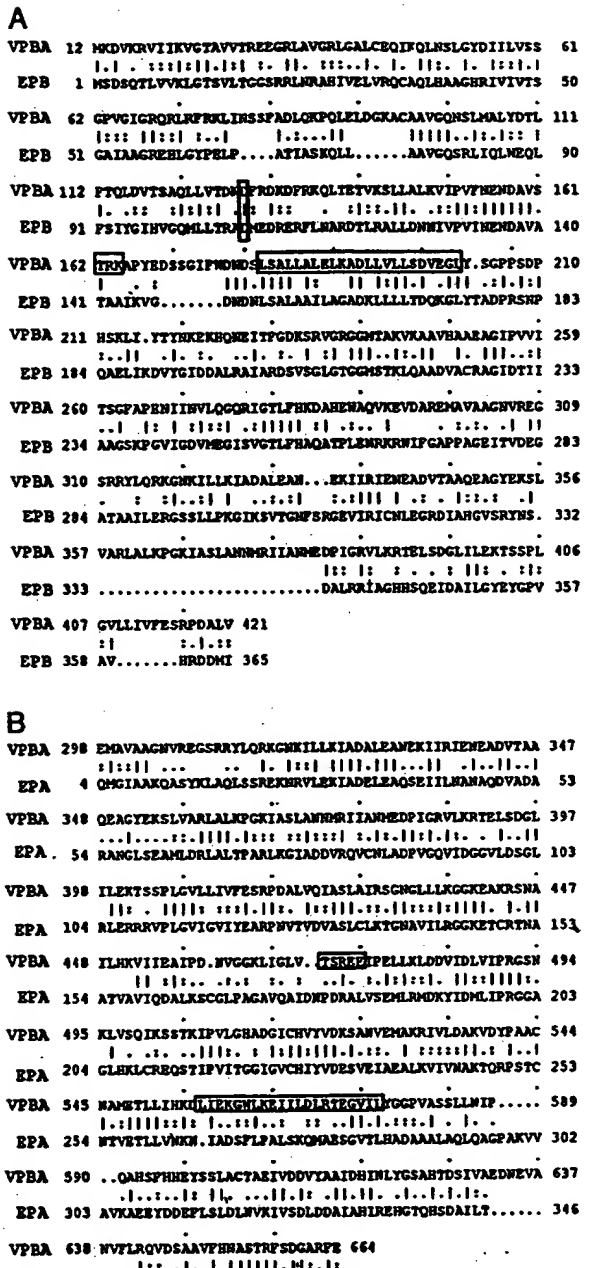

FIG. 2. Comparisons of the *V. aconitifolia* P5CS amino acid sequence (VPBA) with the *E. coli* γ-GK (EPB) (*A*) and GSA dehydrogenase (EPA) (*B*) sequences (10). Identical amino acids are indicated by a vertical line; similar amino acids are indicated by colons and periods. Conserved aspartic acid residues implicated in proline feedback inhibition are boxed, as are the leucine zipper sequences and the potential phosphorylation sites.

than the mutated γ-GK enzyme. The mutation responsible for the resistance to end-product inhibition of γ-GK in the *proB74* allele involves a nucleotide substitution of an A for a G, resulting in a change from aspartic acid to an asparagine residue (29, 33). We note that this aspartate residue is conserved in the *Vigna* P5CS enzyme, raising the possibility that changing this residue to asparagine by site-directed mutagenesis may result in further diminution of feedback

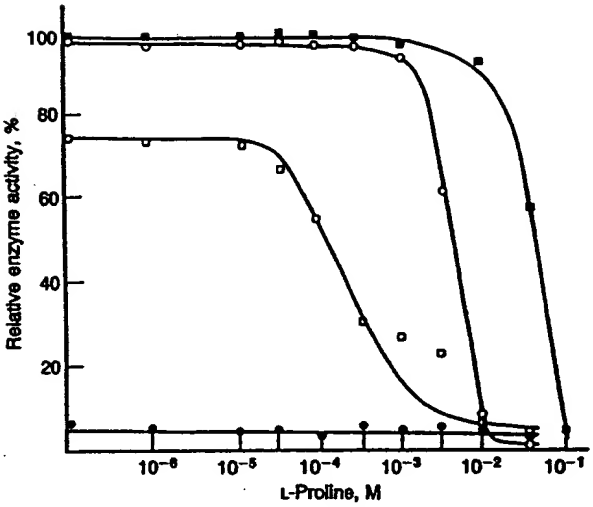

FIG. 3. Feedback inhibition of P5CS by proline. Ammonium sulfate-fractionated extracts (26, 29) from *E. coli* CSH26 (●), *E. coli* CSH26 harboring pVAB2 (○, the *proB74* allele (■), and the wild-type *proB* allele (□) were assayed for γ-GK activity by the method of Hayzer and Leisinger (26).

inhibition of the plant enzyme. In spite of the conserved aspartic acid residue implicated in feedback control, the *Vigna* enzyme is less sensitive to inhibition by proline than the *E. coli* enzyme, suggesting that other regions of the P5CS enzyme may also be important for feedback regulation.

By analogy with the proline biosynthetic pathway in *E. coli* and given the sensitivity of the mothbean enzyme to end-product inhibition, it seems likely that regulation of the pathway in plants is exerted primarily at the P5CS step. The synthesis of P5C in animals, however, is not inhibited by L-proline but is sensitive to ornithine (12–14). In this regard, the plant enzyme is more similar to that in prokaryotes. However, P5C can be made from ornithine in *Vigna* (unpublished data), and therefore the relative contribution of glutamate or ornithine to proline may depend on the availability of nitrogen.

Proline Biosynthesis Genes Are Osmoregulated. We have previously demonstrated that expression of the soybean P5CR gene is enhanced at the RNA level in nodules and in osmotically stressed soybean roots (20), a phenomenon recently confirmed in salt-stressed *Arabidopsis thaliana* leaves.§ To investigate the expression levels of the *Vigna* P5CS gene in different tissues and under conditions of osmotic stress, RNA from nodules, leaves, and roots, as well as roots from plants watered with 200 mM NaCl, was probed on a Northern blot with the pVAB2 cDNA. As shown in Fig. 4, the P5CS transcript in *Vigna* leaves is more abundant than in roots and nodules. The level of P5CS transcripts in roots is enhanced by treatment of the plant with 200 mM NaCl. An increase in the level of transcript of P5CS and P5CR during osmotic stress may facilitate proline production from glutamate. However, the P5CR reaction in plants is not normally rate-limiting (34, 35). The elevated level of P5CS may control the flux of glutamate to P5C. Thus, the proline synthesis pathway is not only feedback regulated but also controlled transcriptionally. The latter is also influenced by the level of nitrogen in the cell.

The elevated rate of proline biosynthesis in nodules has been suggested to stimulate ureide synthesis in tropical legumes and to help transfer redox potential from the nodule cytoplasm to the bacteroids (21, 22). Proline may also act as a carbon and nitrogen source for the bacteroids. An addi-

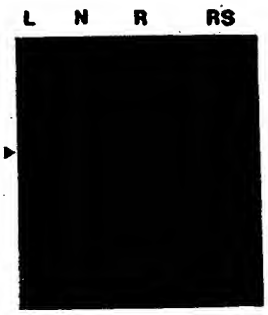

FIG. 4. Northern blot analysis of poly(A)$^+$ RNA (5 μg each) from leaf (lane L), nodule (lane N), root (lane R), and root treated with 200 mM NaCl (lane RS), probed with the ^{32}P-labeled pVAB2 cDNA insert. Arrowhead indicates the 2.6-kilobase P5CS transcript.

tional probable role of proline in nodules may be its involvement in osmoregulation (20). The osmoticum in infected nodule cells is known to be 4- to 5-fold higher than in root cells (36). Our demonstration that both P5CS and P5CR (20) genes are induced by salt stress is consistent with the hypothesis that accumulation of proline in nodules may represent an osmoregulatory adaptation to increased concentration of solutes. Recently, a clone encoding γ-GK has been identified from a tomato cDNA library and is also suggested to contain GSA dehydrogenase activity.[¶] The availability of a cloned plant gene encoding the rate-limiting enzyme in proline biosynthesis will allow understanding of the role of proline in osmoregulation. This opens the way for genetically manipulating this pathway in transgenic plants, with the potential for increasing proline production, which may confer tolerance to drought and salinity stresses in crop plants.

[¶]Garcia-Rios, M. G., LaRosa, P. C., Bressan, R. A., Csonka, L. N. & Hanquier, J. M. Third International Congress, International Society for Plant Molecular Biology, October 6–11, 1991, Tucson, AZ (abstr.).

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Amino Acid and Protein Metabolism in Bermuda Grass During Water Stress[1, 2]

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Received May 31, 1966.

Summary. The ability of Arizona Common and Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] to synthesize amino acids and proteins during water stress was investigated. Amino acids were continually synthesized during the water stress treatments, but protein synthesis was inhibited and protein levels decreased.

Water stress induced a 10- to 100-fold accumulation of free proline in shoots and a 2- to 6-fold accumulation of free asparagine, both of which are characteristic responses of water-stressed plants. Valine levels increased, and glutamic acid and alanine levels decreased.

^{14}C labeling experiments showed that free proline turns over more slowly than any other free amino acid during water stress. This proline is readily synthesized and accumulated from glutamic acid. It is suggested that during water stress free proline functions as a storage compound.

No significant differences were found in the amino acid and protein metabolism of the 2 varieties of Bermuda grass.

In the study of biochemical changes in plants under water stress conditions, increasing attention has been paid to changes in nitrogen compounds. Proteolysis and interruption of protein synthesis are generally found to be results of water stress (6, 11, 20), although both increases and decreases of protein have been found to follow each other (3). Radioisotopes have been used to show the effects of water stress on RNA synthesis and degradation (4). The study presented here reports the effects of water stress on levels and turnover of both free and protein-bound amino acids as shown by ^{14}C labeling.

Water stress induces a characteristic change in the levels of free amino acids, especially a great increase in free proline (3, 6, 12) and amides (3, 9). The accumulation of amides is thought to be the result of incorporation of free ammonia released by deamination of amino acids, which were in turn released by proteolysis induced by water stress (9). Few attempts have been made to explain the accumulation of free proline. The origin and function of this proline is considered in this paper.

Two varieties of Bermuda grass have been used in the present study. These varieties differ somewhat in their general response to water stress, and it was desired to see if under water stress conditions differences also exist in their nitrogen metabolism. An extensive study by Ratnam (13) showed these differences in drought response of Arizona Common and Coastal varieties: Water content and cuticular transpiration are higher in Common Bermuda. Common Bermuda leaves develop a lower (more negative) water potential in a given time without water than do Coastal Bermuda leaves. Leaf damage is generally greater and appears sooner in Common than in Coastal leaves. In general, Ratnam's experimental results tend to support the conclusion that leaves of Coastal are slightly superior to Common in drought avoidance.

Materials and Methods

Plant Material. Clonal material of Arizona Common and Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] was propagated in a 2:1 mixture of sandy loam and sand in 7 inch clay pots. Plants were grown in the greenhouse and were fertilized periodically with commercial fertilizer. The grasses were transplanted into new soil-sand mixture when growth ceased to be vigorous. Tops were cut off periodically. Experiments were conducted in fall or winter, when growth was slow and there was no flowering.

Water Potential Measurement. Water potential was measured with the thermocouple psychrometer device described by Boyer (2).

Labeling of Plants with $^{14}CO_2$. Labeling ex-

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periments were conducted in an isotope hood. Two adjacent rows of plants in clay pots were illuminated from opposite sides with 150-w reflector spot lamps so that shading was minimized. Light was filtered through 9 cm of 0.5 % copper sulfate solution in glass tanks cooled by tap water flowing through copper coils. Light intensity was 1000 ft-c at the leaf surface. Plants were left in place throughout the labeling and sampling periods. Daylength was 8 hours.

For incubation with $^{14}CO_2$, a 15.8 liter bell jar was placed over a plant. Bell jar and plant rested on a glass plate, to which the bell jar was sealed with silicone grease. A 10 ml beaker containing 200 μc of $NaH^{14}CO_3$ (specific activity 25 μc μmole^{-1}) was suspended on a wire inside the top of the bell jar. The bell jar top was sealed with polyvinyl chloride film. To generate $^{14}CO_2$, 0.1 ml of 20 % lactic acid was injected through the film into the beaker; the film was immediately sealed with Scotch tape. After one-half hour, 0.5 ml of concentrated NaOH was injected into the beaker. The bell jar was removed five minutes later. The maximum concentration of CO_2 generated was 0.0012 %, which is small compared to the normal concentration of CO_2 in air. No artifacts due to high CO_2 concentration were likely to have been induced.

Extraction of Free Amino Acids. Plant tissue was killed by boiling it for 3 minutes in 80 % (v/v) ethanol. Tissue and ethanol were stored at −20°. The ethanol was subsequently decanted and saved, and the tissue was ground with mortar and pestle with acid-washed sand and fresh 80 % ethanol. The homogenized sample was refluxed 15 minutes on a steam bath. The sample was centrifuged 15 minutes at 27,000 g. The supernatant fraction was added to the original ethanol in which the tissue was killed. The pellet was refluxed again in 40 % ethanol. This procedure of refluxing and centrifuging was done 4 times in all, once with 80 % ethanol, twice with 40 % ethanol, and once with water. All supernatant fractions were pooled. Four extractions yielded 94 % of the free amino nitrogen obtained in 6 extractions (80 % ethanol, twice in 40 % ethanol, 3 times in water). Pooled extracts were further purified by evaporation almost to dryness at 45° under reduced pressure, taking up the residue in 2 ml of 0.1 N HCl, and centrifuging the suspension 10 minutes at 1° at 27,000 g. This procedure was repeated once or twice; the pellet was discarded each time. Extracts were then purified by the cation exchange method of Wang (18). Recovery of free amino nitrogen in this method was 91 %. This solution was reduced to dryness and the residue was taken up in a small amount of 0.1 N HCl.

Analysis of Amino Acids. Amino acids were measured on an automatic amino acid analyzer using the 1-column technique and buffer sequence of Piez and Morris (10). The analyzer was calibrated with standard mixtures of amino acids. At the column temperature of 60°, glutamine is cyclized to pyrrolidone carboxylic acid, which does not react with ninhydrin. Consequently sample glutamine was not measured. Radioactivity of the analyzer stream was monitored continuously with a Packard 317 scintillation detector and 320E pulse height analyzer. One channel of the amino acid analyzer recorder was used to record radioactivity.

Extraction of Soluble Protein. One grass shoot (up to 15 cm high and 0.5 g dry weight) was cut into 1.5 cm segments and ground with 2 ml water and acid-washed sand with mortar and pestle at 1°. The homogenate was centrifuged at 27,000 g at 1° for 10 minutes. The supernatant fraction was saved. The pellet was ground again with water and sand at 1° and recentrifuged. The water soluble protein in the combined supernatant fraction was precipitated by adding an equal volume of 20 % trichloroacetic acid (TCA) and allowing to settle at least 10 minutes. The protein was centrifuged at 27,000 g for 10 minutes, the pellet was resuspended in 10 % TCA and recentrifuged; then the supernatant was discarded. The pellet was decolorized by twice incubating at one-half hour at 37° with 2 ml of a 2:2:1 (v/v/v) mixture of ethanol, ether, and chloroform, and centrifuging each time. The protein precipitate was dissolved overnight in 1 ml of 1 N NaOH.

Protein Measurement. Protein was measured both by the method of Lowry et al. (8) and by summing the amino acids in protein hydrolysates as measured on the analyzer.

Hydrolysis of Protein. Protein solutions were made to 6 N HCl in 2-piece hydrolysis tubes. After evacuation of air, the solutions were hydrolyzed at 110° for 20 hours. The small amount of humic acid formed was removed by filtration. Hydrolysates were dried on a flash evaporator at 45°. Water was added to the residue and the sample was redried repeatedly to remove excess HCl. The hydrolysate was dissolved in 1 ml 3 M citric acid for analysis on the amino acid analyzer.

Results

In addition to the water soluble protein amino acids, several other amino acids were detected by column chromatographic analysis of ethanolic extracts of Bermuda grass tops. On the basis of elution time and comparative color yield (blue/yellow absorption) the non-protein amino acids α-aminobutyric acid, β-alanine, and pipecolic acid were tentatively identified. The last 2 were present in minute amounts. Extracts of large samples of whole tops revealed minute quantities of still more unidentified ninhydrin-positive compounds. This is illustrated in figure 1 where many small unidentified peaks are shown. Two such compounds were present, however, in amounts large enough to measure. One, labeled N throughout

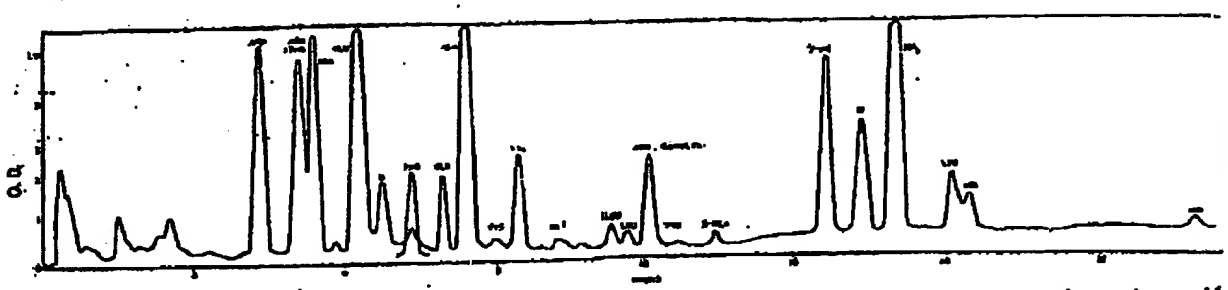

FIG. 1. Chromatogram trace of free amino acids of Bermuda grass produced by the automatic amino acid analyzer. Norleucine (4 μmoles) was added to the sample of fresh tops as an internal standard.

this study, was eluted between glutamic acid and proline. It emerged from the column about 15 minutes after standard citrulline, and disappeared upon hydrolysis with acid.

The other unknown, labeled U, was eluted between α-amino-butyric acid and ammonia. It emerged from the column at the same time as standard ethanolamine and δ-*allo*-hydroxylysine. It was stable to acid hydrolysis. In an attempt to identify this unknown, 2.4 ml fractions were collected during an amino acid analysis of an extract of 136 g fresh Coastal Bermuda grass tops. The solutions in the 3 tubes containing the unknown U were pooled and deionized on a 0.9 × 10 cm column of Dowex 50 × 8, NH_4 form (7). The ammonia solution of the unknown was dried at 40° under reduced pressure. The residue was dissolved in 1 ml 0.1 N HCl. Two 1-dimensional chromatograms were run, using 50 μl of unknown, plus standards, on Whatman No. 1 chromatography paper. Solvent systems were 71 % phenol, and *n*-butanol-propionic acid-water [45.3 : 22.5 : 32.2, v/v/v (1)]. The unknown chromatographed the same distance as ethanolamine in both systems (phenol-water: unknown R_F .70, ethanolamine R_F .68; *n*-butanol-propionic acid-water: unknown R_F .55, ethanolamine R_F .52). The unknown U was therefore tentatively identified as ethanolamine.

An experiment was conducted to determine the effects of water stress on the composition and turnover of both free and protein-bound amino acids. Duplicate sets of both Arizona Common

Table I. *Effect of Water Stress on Fresh Weight, Dry Weight, Total Free Amino Acids, and Water-soluble Protein in Bermuda Grass Shoots*

Treatment	Water potential range, bars	Fr. wt, mg*	Dry wt, mg*
Common			
Control	−4.1 to −7.9	228.6 ± 102.9	46.3 ± 23.2
Moderate stress	−10 to <−37	174.4 ± 69.2	67.6 ± 25.9
Severe stress	<−37	84.6 ± 36.1	61.3 ± 28.6
Coastal			
Control	−4.1 to −4.7	341.3 ± 116.3	69.4 ± 24.2
Moderate stress	−18 to <−37	179.6 ± 48.6	52.4 ± 13.9
Severe stress	−33 to <−37	128.9 ± 61.7	82.6 ± 29.7

Total free amino acids, μmoles per shoot*	Water-soluble protein: μmoles hydrolyzed amino acids per shoot*	mg/shoot	Total amino N, μmoles per shoot
8.72 ± 3.49	18.9** ± 3.5	3.06	27.6
13.9 ± 5.71	12.6 ± 4.7	2.11	26.5
16.7 ± 4.3	9.31 ± 3.41	0.95	25.0
9.26 ± 2.68	23.7 ± 11.4	3.24	33.0
20.4 ± 7.8	13.5 ± 5.3	2.07	33.9
28.0 ± 9.8	9.31 ± 4.13	1.07	37.3

* Average and standard deviation of 5 determinations made in a 77-hour period.
** Four determinations only.

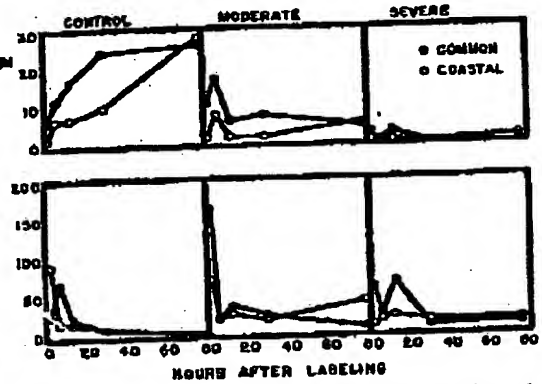

FIG. 2. Time course of change in radioactivity of the free amino acid fraction and soluble protein fraction in Bermuda grass with increasing water stress.

and Coastal Bermuda grass plants in pots were placed under lights in the isotope hood at the beginning of the water stress treatment. One-half of the plants were to be labeled with $^{14}CO_2$. The other half were given the same water stress treatments as the labeled plants but were used for fresh and dry weight measurements and water potential measurements. All stolons and branched shoots were removed. Each plant consisted of 15 to 20 upright shoots 10 to 15 cm high. Water was withheld from treated plants, and controls were watered daily. Water potential was measured daily using as a sample 1 shoot from an unlabeled duplicate plant. It was desired to label plants at each of 2 stress levels which were arbitrarily set at approximately −15 bars (moderate stress) and −30 bars (severe stress) respectively. These stress levels were

Table II. *Changes in Amounts of Free Amino Acids in Bermuda Grass Shoots with Increasing Water Stress*

Amino acid	Control	Moderate stress	Severe stress
	μ moles/gram dry weight**		
Common			
Aspartic acid	11.8 ± 8.9	4.5 ± 1.8	8.4 ± 3.5
Asparagine; threonine	24.6 ± 4.1	29.8 ± 13.7	64.2 ± 17.1
Serine	9.9 ± 2.3	8.3 ± 2.7	11.0 ± 4.5
Glutamic acid	28.7 ± 9.2	10.5 ± 4.8	4.7 ± 1.9
N	...	...	0.8 ± 0.3
Proline	< 2.7	30.5 ± 23.9	69.3 ± 33.0
Glycine	1.8 ± 1.3	1.7 ± 1.1	1.2 ± 0.7
Alanine	31.9 ± 12.3	15.2 ± 3.8	11.6 ± 4.2
1/2-Cystine	...	...	0.6** ± 0.1
Valine	2.1 ± 0.7	3.5 ± 1.6	7.0 ± 2.1
Isoleucine	...	0.9** ± 0.4	1.2 ± 0.4
γ-Aminobutyric acid	3.2 ± 2.1	7.0 ± 4.1	4.3 ± 1.4
U	...	0.8 ± 0.2	1.5 ± 0.9
Ammonia	94.3 ± 36.6	78.0 ± 54.0	55.4 ± 26.4
Lysine	0.5 ± 0.4	0.7 ± 0.1	1.0 ± 0.4
Histidine	...	0.5 ± 0.1	1.4 ± 0.5
Arginine	...	0.8 ± 0.3	2.5 ± 0.1
Totals	211.5	192.9	246.5
Coastal			
Aspartic acid	7.0 ± 5.9	9.0 ± 4.2	9.7 ± 1.7
Asparagine; threonine	9.4 ± 3.3	60.1 ± 21.5	62.5 ± 29.9
Serine	7.9 ± 1.3	18.5 ± 7.9	13.4 ± 6.3
Glutamic acid	22.3 ± 7.7	17.7 ± 6.5	5.4 ± 2.3
N	0.9 ± 0.1	1.3 ± 0.5	0.7 ± 0.3
Proline	< 1.1	138.0 64.0	126.0 34.0
Glycine	0.8 ± 0.2	2.7 ± 1.3	1.8 ± 0.4
Alanine	21.4 ± 6.9	17.3 ± 4.9	13.1 ± 6.3
1/2-Cystine	...	0.8**	0.5** ± 0.1
Valine	1.3 ± 0.2	12.1 ± 3.7	8.4 ± 3.2
Isoleucine	...	2.5 ± 1.9	1.6 ± 0.7
γ-Aminobutyric acid	4.5 ± 3.7	8.4 ± 3.2	4.7 ± 0.9
U	...	1.8 ± 0.4	1.4 ± 0.5
Ammonia	50.8 ± 17.3	81.5 ± 25.6	48.5 ± 6.2
Lysine	0.4	2.2 ± 0.8	1.3 ± 0.4
Histidine	...	1.7 ± 0.1	1.3 ± 0.2
Arginine	...	1.7 ± 0.5	1.8 ± 0.7
Totals	128.0	377.4	302.5

* Average and standard deviation of 4 analyses of common control shoots, 5 of all others. The shoots were each up to 15 cm long and weighed approximately 0.5 g.

** Three or 4 determinations only.

reached at 5 and 7 days without water, respectively. On these days separate plants of each variety were labeled with 200 μc $^{14}CO_2$ as described above. Separate shoots were excised from each of the labeled plants for measurement of free amino acids and of protein-bound amino acids at 1, 5, 12, 29, and 77 hours after the end of the labeling period. Fresh weight, dry weight, and water potential were measured on shoots from unlabeled duplicate plants every day during the 77-hour sampling period after labeling.

A summary of the measurements made is given in table I. During the 77-hour sampling period, water stress was increasing. The highest water potential measurement given was made at the time of labeling, and it decreased thereafter. Therefore all averages of measurements made during the sampling period apply to the entire period when water potential was changing, and not to any average water potential.

Total free amino acids doubled in Common and tripled in Coastal shoots (table I) at maximum water stress employed. At the same time, water-soluble protein decreased in stressed shoots to less than half that of controls. The sum of free and protein-bound amino acids for each variety remained almost constant among all treatments.

The time course of changes in ^{14}C labeling of the total free and water-soluble protein-bound amino acids is shown in figure 2. Initial incorporation of ^{14}C was greatest into, and the most label was retained in, the free amino acid fraction from moderately stressed shoots. Incorporation of label into the free amino acid fraction of severely stressed shoots was not as great as for moderately stressed shoots, but again more label was retained than in controls. Greater treatment differences were found in the incorporation of label into protein. Controls accumulated label into protein continuously during the sampling period. Label in protein from moderately stressed shoots reached a maximum after 5 hours and declined thereafter. At the 77th hour the amount of label in protein from moderately-stressed shoots was only 20 % that

Table III. *Effect of Water Stress on Protein Composition*

Average and standard deviation of 4 measurements for Common control, 5 for all others. Cystine and tryptophan not determined and not included in calculations. Amide N not determined.

Amino acid	Control	Mole percent Moderate stress	Severe stress
Common			
Aspartic acid	10.1 ± 1.5	9.0 ± 1.2	9.1 ± 1.1
Threonine	4.9 ± 0.5	4.0 ± 0.3	4.4 ± 0.4
Serine	4.9 ± 0.2	4.7 ± 0.3	5.1 ± 0.6
Glutamic acid	11.0 ± 0.6	11.5 ± 0.4	11.5 ± 0.8
Proline	5.5 ± 0.3	6.0 ± 0.4	5.5 ± 0.7
Glycine	9.7 ± 0.4	11.0 ± 0.7	11.0 ± 0.7
Alanine	9.9 ± 0.5	10.9 ± 0.4	10.9 ± 0.3
Valine	8.3 ± 0.5	8.5 ± 0.4	8.4 ± 0.6
Methionine	1.4 ± 0.6	1.6 ± 0.6	1.7 ± 0.3
Isoleucine	5.5 ± 0.6	6.2 ± 1.0	5.4 ± 0.2
Leucine	9.1 ± 0.6	9.6 ± 0.4	9.3 ± 0.5
Tyrosine	3.1 ± 0.2	3.9 ± 0.3	3.1 ± 0.4
Phenylalanine	4.1 ± 0.2	4.2 ± 0.2	4.1 ± 0.3
Lysine	6.3 ± 0.5	5.7 ± 0.3	6.3 ± 0.8
Histidine	1.8 ± 0.2	1.6 ± 0.3	1.7 ± 0.3
Arginine	4.5 ± 0.5	2.7 ± 0.6	3.1 ± 0.5
Coastal			
Aspartic acid	9.2 ± 0.5	9.2 ± 0.9	9.7 ± 0.5
Threonine	4.6 ± 0.3	4.1 ± 0.3	4.2 ± 0.4
Serine	5.0 ± 0.2	4.8 ± 0.3	5.4 ± 1.0
Glutamic acid	11.1 ± 0.6	11.6 ± 0.3	11.4 ± 0.9
Proline	5.9 ± 0.7	5.8 ± 0.5	5.4 ± 0.9
Glycine	9.9 ± 0.4	11.1 ± 0.5	10.7 ± 0.5
Alanine	10.2 ± 0.3	10.6 ± 0.5	10.9 ± 0.5
Valine	8.2 ± 0.2	8.5 ± 0.3	8.7 ± 0.2
Methionine	1.7 ± 0.4	1.9 ± 0.1	1.6 ± 0.5
Isoleucine	5.8 ± 0.2	5.5 ± 0.3	5.3 ± 0.3
Leucine	9.3 ± 0.2	9.6 ± 0.3	9.1 ± 0.7
Tyrosine	3.1 ± 0.3	2.9 ± 0.2	2.7 ± 0.3
Phenylalanine	4.4 ± 0.7	4.1 ± 0.2	3.9 ± 0.3
Lysine	6.0 ± 0.8	5.6 ± 0.5	6.2 ± 0.9
Histidine	1.8 ± 0.2	1.8 ± 0.3	1.7 ± 0.2
Arginine	3.9 ± 0.6	2.9 ± 0.5	3.1 ± 0.5

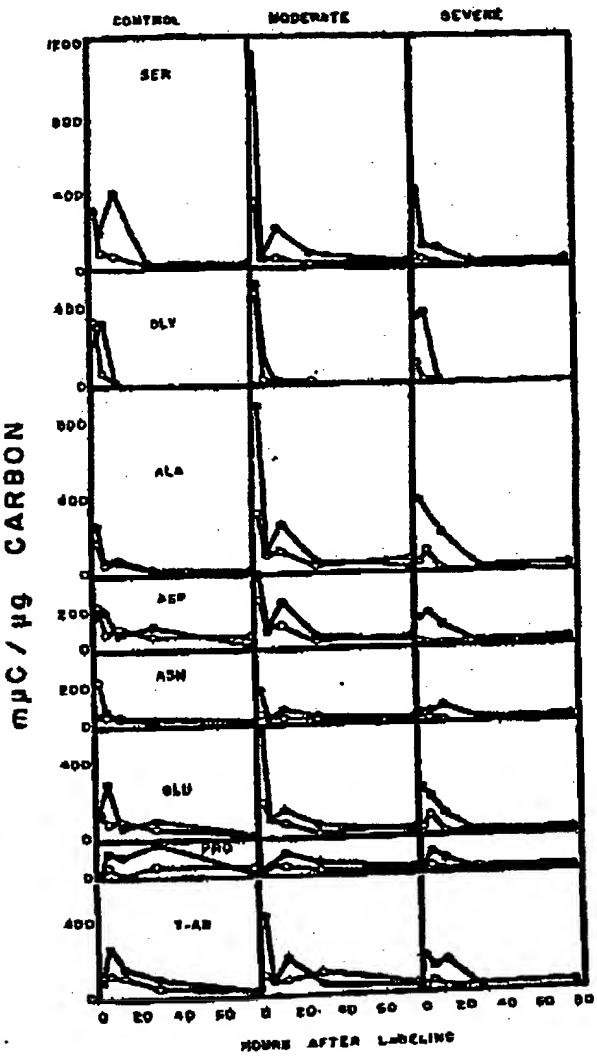

FIG. 3. Changes in specific activity of individual free amino acids from $^{14}CO_2$-labeled Bermuda grass shoots.

of controls. Very little label was incorporated into protein from severely stressed shoots. These data support the conclusion that free amino acids are readily synthesized during water stress, but are not as readily incorporated into protein as in unstressed controls.

Changes in amounts of individual free amino acids with increasing water stress are shown in table II. The free amino acid concentrations in control shoots of both varieties were similar except that Common shoots contained more than twice as much free asparagine as Coastal shoots. From comparison of the blue-yellow light absorption ratios for the ninhydrin reaction products of asparagine and threonine, and from preliminary analyses of mild acid hydrolysates of purified free amino acid extracts, it was found that the asparagine content of shoots is much greater than the threonine content. Therefore the combined asparagine-threonine peak was calculated as asparagine. The increase in this peak during water stress was also due to increased asparagine content. Free proline concentration increased dramatically during water stress to 10 to 125 times its control value. At the same time, valine content tripled, and glutamic acid and alanine concentrations decreased.

Serine, glycine, and alanine were the free amino acids that became most highly labeled (fig 3). Aspartic acid, glutamic acid, γ-aminobutyric acid, and asparagine all incorporated somewhat less label. All of the free amino acids found in the controls except proline became more highly labeled in the moderately stressed shoots than in any other treatment or the controls. Proline seemed to become labeled slowly. The low specific activity of proline obscures the fact that the great amount of proline present in stressed plants contained more than half the activity remaining in the free amino acid fraction after 77 hours. The irregularities in changes in specific activities of the individual amino acids occur for all the amino acids of a specific sample. This indicates that the differences reside in the level of labeling of individual shoots, and not that specific activities changed irregularly within any 1 shoot.

Changes in the amino acid composition of water-soluble protein with increasing water stress are given in table III. Conditions of hydrolysis did not permit preservation of cystine or tryptophan; consequently these had to be omitted from the calculations. The largest change in protein composition in stressed shoots was the 20 to 40 % decrease in arginine content. There was also a small decrease in threonine content.

Changes in the specific activity of individual protein-bound amino acids are shown in figure 4. The higher specific activity in Common shoots is attributed to the smaller size of Common shoots, which results in a concentration of label when plants were exposed to equal amounts of label. The specific activity curves for most protein amino acids from control or moderately stressed shoots level off after 5 or 12 hours. Specific activity of protein amino acids from severely stressed shoots was considerably lower than in the other treatments. Generally maximum specific activity was achieved after 1 to 12 hours, followed by a sharp decline. The specific activity curves, together with the protein data of table I, are interpreted to mean that there is a net loss of protein during water stress although some synthesis occurred.

To test whether or not proline could be synthesized from glutamic acid (17) in stressed plants, control and stressed shoots were incubated with glutamic acid-U-^{14}C. Two well-watered control shoots and 2 shoots from a Coastal plant not watered for 6 days were excised and quickly placed in 0.05 ml of water contained in 2 cm conical centrifuge tube tips. The shoots were illuminated as before.

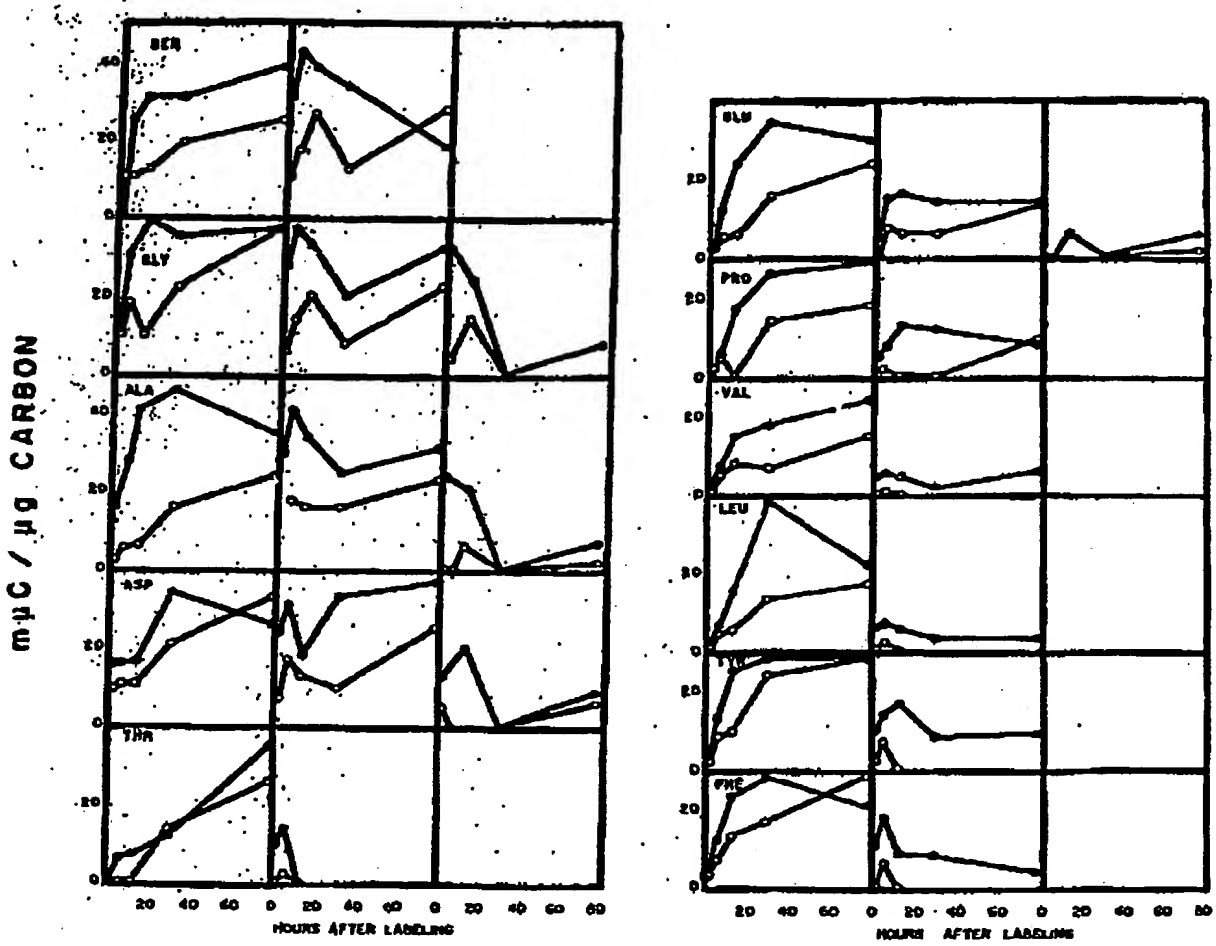

FIG. 4. Changes in specific activity of individual soluble protein amino acids from $^{14}CO_2$-labeled Bermuda grass shoots. Specific activities were calculated from average protein composition values for Common Bermuda controls and the measured radioactivity figures.

To the water was added 3.0 μc of randomly labeled ^{14}C-glutamic acid, monoammonium salt, specific activity 10 mc mmole^{-1}. Water was added to the vessels in 0.02 ml increments to replace that taken up by the shoots. One control and 1 stressed shoot were killed in boiling 80 % ethanol after 1 hour; the other 2 shoots were killed after 3 hours. Amino acid extracts were made in the usual manner, except that the ion exchange purification step was omitted. Radioactivity and ninhydrin-positive compounds were measured on the analyzer.

Results are shown in table IV. About half of the amino acids, plus at least 11 ninhydrin-negative compounds, became labeled both in control and stressed shoots. Proline was very slightly labeled in controls, but specific activity was fairly high because of the low amount present. The proportion of recovered label in proline from controls was less than 1 % at both sampling times, whereas this proportion in stressed plants was 6.0 % at 1 hour and somewhat less than 8.6 % at 3 hours. The actual activity in proline was 25 and 16 times greater in stressed plants than in controls at 1 and 3 hours respectively. It is concluded that water-stressed Bermuda grass shoots convert glutamic acid to proline, and accumulate the newly synthesized proline, much more readily than do well-watered shoots. Turnover of new proline was also very slow; labeled proline was still being accumulated 3 hours after labeling.

The data of table IV also show that glutamic acid-U-^{14}C disappears at about equal rates from stressed and control shoots. The largest amounts of label were recovered in several ninhydrin-negative peaks eluted before aspartic acid. These peaks represent sugars and organic acids which would ordinarily have been lost if the sample had been purified by the ion exchange method.

Table IV. *Distribution of Radioactivity in Soluble Compounds of Coastal Bermuda Grass Shoots Incubated with Glutamic Acid-U-^{14}C*

Compound	Control (−3.5 bars) 1	Control (−3.5 bars) 3	Stressed (−25.2 bars) 1	Stressed (−25.2 bars) 3
1*	...	...	..	67
2	71	...	...	194
3	53	170	...	
4	83	250	...	173
5	83	...	...	
6	120	53	...	101
7	77	53	...	80
8	11	...	...	...
9	12	...	...	14
10	6.5	2.4	...	...
11	10	3	...	5
Aspartic acid	75.5	31	...	67
Asparagine and threonine	19	7.1	14	34
Serine	15	16	12	15
Glutamic acid	268	188	410	192
Proline	2	6.5	50	101
Lysine	...	...	2	...
Alanine	77	28	47	81
12	8	5	...	1
13	40	11	35	28
Valine	1	2	...	...
Isoleucine	1	2	1	1
14	4	...	...	1
Leucine	3	...	2	1
15	2	...	1	2
γ-Aminobutyric acid	91.5	24	63	12
Totals	1130	854	790	1170

* Ninhydrin-negative compounds, numbered in order of elution from the analyzer column, were detected as radioactivity peaks only.

Discussion

Soluble protein levels in Bermuda grass were found to decrease with increasing water stress. Chen et al. (3) have reported successive increase, decrease, and a second increase in protein levels with increasing stress in citrus seedlings. These changes parallel Stocker's (15) activation, reaction, and restitution phases of drought response. The data presented here do not fit this pattern. However, the Bermuda grass data represents certain water stress levels, while the citrus data were taken at strict time intervals after withholding water.

The marked loss of protein-bound arginine in stressed Bermuda grass shoots has not been reported for other plants. This loss may reflect a preferential hydrolysis of arginine-rich protein. Such proteins are found in nuclei (5) and in ribosomes (16). Water stress can induce either an increase (19) or a decrease (14) in ribosomal RNA, but ribosomal proteins and nuclear proteins have not been investigated in connection with water stress. However, basic nuclear and ribosomal proteins as a whole are rich in lysine as well as in arginine, and no such loss in protein bound lysine was detected as a result of water stress. This could be interpreted to mean the loss of protein arginine involves the loss of some arginine-rich but lysine-poor protein. Furthermore, the loss in protein arginine may account for the observed very slight rise in free arginine.

During severe water stress, photosynthesis, starch accumulation, and protein synthesis are all inhibited to some degree. In stressed Bermuda grass shoots enough $^{14}CO_2$ was fixed to label free proline that turned over very slowly. The ^{14}C-glutamic acid labeling data clearly show that stressed shoots readily accumulated much more proline newly synthesized from glutamic acid than do control shoots. The slow turnover of labeled proline may also reflect an inhibition of proline catabolism. Free proline may be acting as a storage compound for both carbon and nitrogen during water stress, when both starch and protein synthesis are inhibited. Such a storage compound might be utilized for growth upon rewatering.

The changes in levels of free amino acids accompanying water stress in Bermuda grass are similar to those found in water stressed citrus

seedlings (3), pumpkin roots (20), and cut ryegrass (6).

Throughout this study, possible differences in nitrogen metabolism between Common and Coastal varieties were sought. During water stress, free proline accumulated to the highest levels in Coastal shoots. Under well watered conditions Common shoots contained the largest amounts of free asparagine. Aside from these minor observations, no differences were detected that might serve as a basis for explanation of the known differences in drought response. Such differences are still best explained on anatomical and morphological grounds (13).

Acknowledgments

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WGK

CHAPTER 9

Betaines

R. G. WYN JONES AND R. STOREY

PHYSIOLOGY AND BIOCHEMISTRY OF DROUGHT RESISTANCE IN PLANTS

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I. INTRODUCTION

A. DEFINITIONS AND NOMENCLATURE

In 1953 Wheeland defined *onium* compounds as "substances formed by an addition reaction, in the course of which some atom increases its valency by one unit and in doing so increases its formal charge, algebraically, by one unit". This definition encompasses a very broad group of compounds of which quaternary ammonium and tertiary sulphonium compounds with fixed positive charges on, respectively, the nitrogen and sulphur atoms are probably the more important biologically. These are widely, but apparently erratically, distributed in nature. Cantoni (1960) noted their greater abundance and diversity in marine invertebrates, fish and certain plants compared with micro-organisms and vertebrates. Nevertheless, specific onium compounds, such as acetylcholine, have a fundamental role in vertebrate biochemistry.

In this chapter our major concern will be *ammonio* compounds (the preferred chemical term), particularly that class of fully N-methyl substituted amino acids to which the trivial name *betaines* is applied. Some attention will also be directed to closely related compounds, for example choline-O-sulphate, and to *sulphonio* analogues of the betaines. In plant biochemistry there is some interest in phosphonium compounds in view of their role as plant growth regulators (Cathey, 1964), but they lie outside the scope of this chapter.

The trivial nomenclature in this field is in great disarray and, with the increasing interest in the physiology and biochemistry of these compounds, the establishment of a clear and agreed system assumes some importance. We will adopt the convention of naming individual betaines by reference to their parent amino acid (e.g. N,N'-dimethylproline, often referred to as stachydrine, will be called prolinebetaine by analogy with the well-established practice of calling N,N',N''-trimethylglycine glycinebetaine). A number of betaines found in plants, together with their trivial names, are recorded in Table I. This system of naming will not be applied to the "betaines" produced by the methylation of the ring nitrogen of pyridine as there is no root amino acid; thus the name trigonelline will be retained (Table I). We feel it is important that the use of "betaine" to describe glycinebetaine be discontinued, despite our own use of the term in early papers and its recent use by others (Hall et al., 1978; Hanson & Nelson, 1978). Using the same term for a class of compounds and for a particular member of that class can only lead to confusion. The term, *thetin*, for methyl-substituted sulphonio compounds will be dispensed with as it is not (as used by Challenger, 1959) formally analogous with "betaine"; the recognized chemical terminology, sulphonium (sulphonio), will be employed.

TABLE I
Names and structures of major betaines.

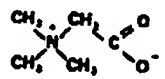
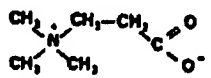
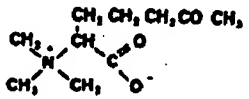
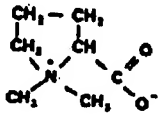
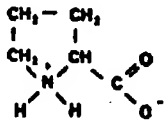
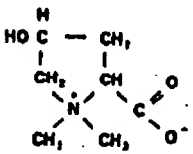
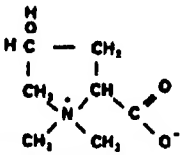
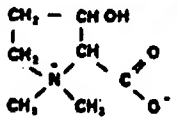
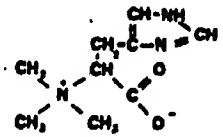
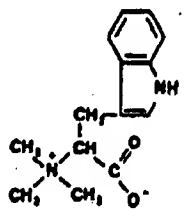
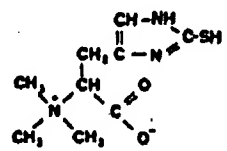
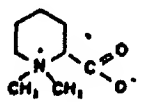
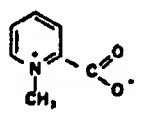
Structures	Preferred trivial names	Other trivial names
	Glycinebetaine	Oxyneurin, Betaine
	β-alaninebetaine	Homobetaine
	2-trimethylamino-6-ketoheptanoate	
	Prolinebetaine	Stachydrine
	Proline	
	(−)4-hydroxyprolinebetaine	Betonicine
	(+)4-hydroxyprolinebetaine	Turicine

Table I continued

Names and Structures of major betaines.

Structures	Preferred trivial names	Other trivial names
	3-hydroxyprolinebetaine	3-oxystachydrine
	Histidinebetaine	Herzynine Ercinine
	Tryptophanbetaine	Hypaphorine
	2-mercaptohistidine-betaine	Ergothioneine
	Pipecolatebetaine	Homostachydrine
	Trigonelline	

8. NATURALLY OCCURRING BETAINES

Of the wide variety of betaines found in plants, glycinebetaine was the first to be isolated and has been subjected to the closest scrutiny. The compound was first isolated by Husemann and Marme in 1863 from the arid zone shrub, *Lycium barbarum*, and was named lycin (see Karrer, 1958). Happily, this and other names found in the early literature (e.g. oxyneurin, glykokollbetaine) have been abandoned. At the turn of the century, the extensive studies of Schulze and Stanek and their colleagues (see Guggenheim, 1958, for references) established that glycinebetaine was found in high concentrations in some plants, particularly *Atriplex* spp. and sugar beet. During this period prolinebetaine and trigonelline were also characterized, as were most of the more obscure betaines noted in Table I. β-alaninebetaine, however, was characterized relatively recently. Interest in sulphonio compounds developed rather later, and the work is described in Challenger's classic book (1959) on organic sulphur chemistry.

Despite the early activity of the German school of natural product organic chemists, little biochemical interest developed in these compounds apart from the recognition of the role of some N-methyl and S-methyl compounds in l-carbon metabolism and methyl donation (Cantoni, 1960). Only recently has an hypothesis been formulated to account for the large quantities of betaines, particularly glycinebetaine, found in certain plants (Storey & Wyn Jones, 1975; Wyn Jones et al., 1977a, 1977b). Glycinebetaine is proposed to be a major cytoplasmic osmoticum in certain higher plant families adapted to salt or water stress. It may be that other betaines and, possibly, methyl sulphonio compounds have a similar function in other species, but in all cases inadequate evidence is available.

In this chapter we will summarize data on the taxonomic and tissue distribution of betaines and will consider their biochemistry and physiology in relation to water and salt stress. It is inevitable that the major emphasis will be on glycinebetaine as less is known about the other compounds. And it is of particular interest to consider whether glycinebetaine accumulation is a positive adaptation to stress or an incidental side reaction. There is extensive literature on betaines and related compounds in invertebrates and, to a lesser extent, marine algae, besides the work of Avi-Dor and his colleagues on a moderately halophilic bacterium.

II. PHYTOTAXONOMIC DISTRIBUTION OF BETAINES

A. GLYCINEBETAINE

Glycinebetaine is structurally the simplest of the betaines; its dipolar structure is given in Table I. Proline is also included in Table I to illustrate the structural similarities of some of the compounds under discussion. As far as is known, glycinebetaine is always present as the free dipole, but this is not the case for some of the other betaines, notably β-alaninebetaine.

In Table II data on the taxonomic distribution of glycinebetaine and some of the other betaines are summarized. Glycinebetaine is widely distributed and often occurs in very large quantities. The data suggest that there is a taxonomic basis to glycinebetaine distribution and almost certainly to its accumulation as a major metabolite. Much more work will be required to establish this firmly and to define which families and species have this capacity.

There are indications in Table II that glycinebetaine accumulation is associated with halophytic and, possibly, xerophytic characteristics and arid and salty habitats. There is, however, a certain distortion inherent in these data. Because glycinebetaine was found in halophytic species, there has been a tendency to concentrate the various surveys on saline and dry habitats, and relatively few glycophytes and mesophytes have been studied. Not all xerophytic or halophytic species accumulate glycinebetaine or other betaines under field conditions, and no salt-sensitive or drought-sensitive plant with a low sap osmotic pressure has been found to accumulate one of these compounds.

Glycinebetaine has also been found in considerable concentrations in the tissues (usually muscle) of marine invertebrates (Beers, 1967; Schoffeniels & Gilles, 1972), in fungi (Ardreeva, 1971), in some marine algae (Takemota & Sai, 1964), and in bacteria (Rafaeli-Eshkol & Avi-Dor, 1968).

B. PROLINEBETAINE AND HYDROXYPROLINEBETAINES

Much attention is focused in this volume on the physiology and biochemistry of proline accumulation in plants subject to water stress. Consequently, it is of particular interest that the betaines of proline and of 3- and 4-hydroxyproline occur in plants (see Table I for nomenclature and structures; see Table II for phytochemical distribution). Of these compounds, prolinebetaine itself appears the most common and is a characteristic product

TABLE II
Distribution of betaines in higher plants

Genus and species	Tissue	Osmotic potential (10^5 Pa)	Division 1	Ontum compound µmol. g dry wt^{-1} or (.g fresh wt^{-1})	Ecology or habitat of collected species	Assay method and reference
			DICOTYLEDONS[a]			
			Lignose (fundamentally woody)			
Order Capparales						
FAMILY CAPPARACEAE						
36 species	leaves		pb	present in 33 species		4Q
Capparis tomentosa	seeds		OH-pb			
			pb		arid zone	4,5 V
Order Euphorbiales						
FAMILY EUPHORBIACEAE						
Euphorbia paralias	leaves	−8.6	nlg		coastal sand dune	1A
Order Leguminales						
FAMILY LEGUMINOSAE						
Acacia brachybotrya	shoots	−27.0	ND		arid sandy	2B
Acacia hakeoides	leaves	−22.3	ND		arid sandy	2B
Acacia linearia	shoots		ND		arid sandy	2B
Acacia sparophylla	shoots	−24.4			arid sandy	2B
Acacia tripera	shoots	−25.3	ND		saline	2B
Cassia artemisioides	shoots	−21.2	nlg		arid sandy	2B
Medicago sativa	shoots	−21.0	pb	(25)	arid sandy	2B
Phaseolus vulgaris	shoots	−11.3	nlg		grown in 200 mmol $NaCl$	1Z
Pisum sativum	shoots	−18.2	nlg	5	grown in 75 mmol $NaCl$	1C,D
Trifolium repens	shoots	−11.3	nlg	5	grown in 100 mmol $NaCl$	1C,D
Vicia faba	detached shoots		pb	125	grown in 75 mmol $NaCl$	1C,D
						3H

Table II continued

Genus and species	Tissue	Osmotic potential (10^5 Pa)	Name	Onium compound μmol. g dry wt^{-1} or (.g fresh wt^{-1})	Ecology or habitat of collected species	Assay method and reference
Order Malvales						
FAMILY MALVACEAE						
Abutilon albescens	leaves	−1.6	gb		arid coral island	2 D
Abutilon otocarpum	leaves	−36.1	gb	213	halophyte	2 B
Lavatera plebeja	shoots	−19.8	D+		arid sandy	2 B
Order Myrtales						
FAMILY MYRTACEAE						
Melaleuca uncinata	shoots	−25.0	D+		arid sandy	2 B
Order Sapindales						
FAMILY SAPINDACEAE						
Dodonaea attenuata	shoots	−23.9	ND		saline	2 B
Heterodendron oleifolium	shoots	−20.2	trig		arid sandy	2 B
Order Verbenales						
FAMILY VERBENACEAE						
Avicennia marina	leaves	−33.8	gb	265	interidal mangrove	2 B

DICOTYLEDONS

Division II Herbaceae (fundamentally herbaceous)

Genus and species	Tissue	Osmotic potential (10^5 Pa)	Name	Onium compound μmol. g dry wt^{-1} or (.g fresh wt^{-1})	Ecology or habitat of collected species	Assay method and reference
Order Asterales						
FAMILY ASTERACEAE (Composite)						
Aster tripolium	shoots	−24.9	gb	164	salt marsh	1 A
	shoots		gb.(P)	(29)	coastal	W
Centaurea melitensis	shoots	−15.4	ND		arid sandy	2 B
Chondrilla juncea	shoots	−14.4	ND		arid sandy	2 B
Erigeron bonariensis	shoots	−19.2	gb	68	salt marsh	2 B
Helichrysum apiculatum	shoots	−16.4	ND		arid sandy	2 B
Lxiolaena leptolepis	shoots	−27.2	ND		arid saline	2 B
Matricaria maritima	shoots	−6.4	gb.D+	55	coastal sand dune	1 A
Minuria leptophylla	shoots	−50.6	gb	175	halophyte	2 B
Myriocephalus stuartii	shoots	−10.0	ND		arid sandy	2 B
Ptachea lanceolata			gb		hot regions of India	4 I
Senecio spathulatus	shoots	−15.3	pb		coastal sand dune	2 F
Sonchus oleraceus	shoots	−21.2	ND		dry saline	2 B
Vittadinia cuneata	shoots	−17.2	gb	42	arid sandy	2 B
Waitzia acuminata	shoots	−15.9	ND		dry saline	2 B
Wedelia biflora	leaves	−9.3	dsp		dry coral island	2 E
Order Brassicales						
FAMILY CRUCIFERAE						
Cakile maritima	shoots	−14.9	ND		sand dune	1 A
Cochlearia officinalis	shoots		gb.(P)	trace	coastal	W
Raphanus sativa	shoots		ND		grown in 100 mmol NaCl	2 D
Order Caryophyllales						
FAMILY AIZOACEAE						
Mesembryanthemum crystallinum	shoots		ND		200 mmol NaCl	2 Z
Psilocaulon (Haw) Schwantes	shoots	−38.3	2 D+		saline	2 B
Tetragonia expansa	tops		ND			5 J
FAMILY CAROPHYLLACEAE						
Spergularia marina	shoots		gb.(P)	trace	coastal	W
Spergularia media	shoots		gb.(P)	trace	coastal	W
Order Chenopodiales						
FAMILY CHENOPODIACEAE						
Arthrocnemum halocnemoides	shoots	−59.1	gb	280	halophyte	2 B
Atriplex canescens	leaves		gb	279	halophyte	3 H
	leaves		gb	323	halophyte	5 J
Atriplex halimus	leaves		gb	418	halophyte	3 H
Atriplex hastata	shoots	−18.1	gb	75	halophyte	2 B

Table II continued

Atriplex hortensis			gb	84-107		3 H
Atriplex inflata	shoots	−34.7	gb	223	halophyte	2 B
Atriplex nummularia	leaves	−37.7	gb	152	halophyte	2 B
Atriplex patula	leaves		gb	112-225	halophyte	3 H
Atriplex pseudocampanulata	shoots	−40.2	gb	236	halophyte	2 B
Atriplex rosea	shoots		gb	94		5 J
Atriplex semibaccata	shoots	−35.6	gb	249	halophyte	2 B
Atriplex spongiosa	leaves	−34.7	gb	340	halophyte — grown in 500 mmol NaCl	2 C,D
Atriplex suberecta	leaves	−13.9	gb	260	saline	2 B
Atriplex vesicaria	shoots	−62.0	gb	251	halophyte	2 B
Atriplex wootonii	leaves		gb	284		5 J
Babbagia acroptera	shoots	−43.6	gb	187	halophyte	2 B
Bassia brachyptera	shoots	−43.2	gb	179	halophyte	2 B
Bassia intricata	shoots	−48.1	gb	214	halophyte	2 B
Bassia lanicuspus	shoots	−31.2	gb	272	halophyte	2 B
Bassia parviflora	shoots	−27.1	gb	169	halophyte	2 B
Bassia patenticuspis	shoots	−31.3	gb	168	halophyte	2 B
Bassia quinquecuspis	shoots	−32.0	gb	171	halophyte	2 B
Bassia stelligera	shoots	−22.3	gb	193	arid saline	2 B
Bassia tricuspis	shoots	−51.9	gb	205	halophyte	2 B
Beta cycla	leaves		gb	287		5 J
Beta maritima	leaves		gb	195	salt marsh	5 J
Beta trigyna	leaves		gb	179		5 J
Beta vulgaris	leaves		gb	223	salt resistant	5 J
Chenopodium album	leaves		gb	104		5 J
Chenopodium bonus-henricus	leaves		gb	190		3 H
Chenopodium botrys			gb	129		4 K
Chenopodium foetidum	whole plant		gb	123	sea-shore	5 J
Chenopodium nitrariaceum	shoots	−32.9	gb	173	halophyte	2 B
Chenopodium pseudomicrophyllum	shoots	−33.4	gb	92	halophyte	2 B
Chenopodium urbicum	shoots		gb	37		5 J

Chenopodium vulvaria	leaves		gb	158		3 H
	whole plant		gb	97		5 J
Corispermum marschellic	shoots		gb	12		5 J
Corispermum tamnoides	shoots		gb	35		5 J
Enchyloena tomentosa	shoots	−31.0	gb	216	halophyte	2 B
Halbirzia tamnoides	whole plant		gb	85		5 J
Halimone portulacoides	shoots	−20.5	gb	238	frost resistant salt marsh	1 A
Kochia trichophylla	whole plant		gb	81		5 J
Kochia scoparia	whole plant		gb	189		5 J
Maireana sp.	shoots	−28.8	gb	323	halophyte	2 B
Maireana appressa	shoots	−39.4	gb	269	halophyte	2 B
Maireana pyramidata	shoots	−31.8	gb	278	halophyte	2 B
Malacocera tricorne	shoots	−42.7	gb	36	halophyte	2 B
Orbione sibirica	shoots		gb	153	salt marsh	5 J
Pachycornia tenuis	shoots	−59.2	gb	533	halophyte	2 B
Rhagodia spinescens	shoots	−32.3	gb	152	halophyte	2 B
Salicornia quinqueflora	shoots	−30.7	gb	181	wet saline	2 B
Salicornia fruticosa	shoots		gb	174	wet saline	4 L
Salicornia europaea	shoots		gb	(45)	coastal	W
Salsola kali	shoots		d	81	coastal dunes	5 J
Salsola kali, tetrandra longifolia and *rigida*			gb	present in all 4		
Spinacia oleracea	leaves		gb	23	salt resistant	4 M
	leaves		gb	162	salt resistant	3 H
Suaeda maritima	shoots		gb	(63)	wet saline	5 J
Suaeda monoica	leaves	−53.1	gb	340	wet saline	2 W,X
Threlkeldia salsuginosa	shoots	−29.7	gb	203	halophyte	2 C,D
FAMILY AMARANTHACEAE						2 B
Achyranthes aspera	whole plant		gb	22		
Achyranthes aspera	shoots	−7.0	gb		dry coral island	6 N
Amaranthus caudatus	leaves		gb	46		2 E
Amaranthus caudatus	leaves		gb	186		3 H
Amaranthus retroflexus	whole plant		gb	82		5 J
						5 J

Table II continued

Order Lamiales						
FAMILY LABIATAE						
Ajuga australis	shoots	−14.1	ND		arid sandy	2 B
Eremostachys speciosa			pb	25		O
Lamium album			pb	140		O
Lagochilus hirtus			pb	57		3,4 P
Lagochilus inebrians			pb	36		O
Lagochilus platycalyx			pb	101		O
Lagochilus pubescens			pb	50		O
Lagochilus setulosus			pb	91		O
Leonurus turkestanicus			pb	105		O
Marrubium vulgare			OH-pb	19		6 Q
Marrubium vulgare	shoots	−18.4	D+		arid sandy	2 B
Sideritis montana			pb	57		O
Stachys betonicaeflora			pb	52		O
Stachys hissarica			pb	33		O
FAMILY MYOPORACEAE						
Eremophila mitchelli	shoots	−19.3	D+		arid sandy	2 B
Order Polygonales						
FAMILY POLYGONACEAE						
Muehlenbeckia cunninghamii	shoots	−22.1	trig		arid saline	2 B
Polygonum aviculare	shoots		ND			5 J
Polygonum divaricatum	shoots		ND			5 J
Order Plantaganales						
FAMILY PLANTAGINACEAE						
Plantago coronopus	shoots		ND		coastal	W
Plantago maritima	shoots		ND		coastal	1 A,W
Order Primulales						
FAMILY PLUMBAGINACEAE						
Armeria maritima	shoots	−19.8	β-ab.(P)		salt marsh	1 A,Y

Limmonium vulgare	shoots	−24.7	β-ab.(P)		salt marsh	1 A
Limmonium vulgare			β-ab.(P).tmkh	50	halophyte	4,6 R,S
Plumbago capensis	shoots		ND			Y
FAMILY PRIMULACEAE						
Glaux maritima	shoots		gb.(P)	trace	coastal	W
Order Solanales						
FAMILY CONVOLVULACEAE						
Evolvulus alsinoides	whole plant		gb			4,6 T
FAMILY SOLANACEAE						
Cestrum parqui	leaves	−13.3	D+		arid sandy	2 B
Lycium barbarum	whole plant		gb	333		5 J
Lycium chinense	leaves		gb	166		3 H
Lycium ferocissimum	leaves	−24.5	gb	150	saline	2 B
Lycopersicon esculentum	leaves		trig	0.5	100 mmol NaCl	1 C,D
Nicotiana velutina	leaves	−15.3	ND		saline	2 B
Solanum esuriale	shoots	−16.1	trig		arid sandy	2 B
Order Umbellales						
FAMILY UMBELLIFERAE						
Daucus carota	shoots	−18.1	ND		100 mmol NaCl	1 D
Eryngium maritimum	shoots		ND		coastal	W

MONOCOTYLEDONS

Genus and species	Tissue	Osmotic potential (10^5 Pa)	Onium compound Name	μmol. g dry wt^{-1} or (.g fresh wt^{-1})	Ecology or habitat of collected species	Assay method and reference
Order Graminales						
FAMILY GRAMINEAE						
Festuceae (Tribe)						
Festuca rubra	shoots		gb.(P)	(19)	coastal	W
Puccinellia distans	shoots		gb.(P)	trace	coastal	W

Table II continued

Puccinellia maritima	young leaves	−22.7	gb,(P)	2.6	salt marsh	1 A,W
Agrosteae						
Agrostis stolonifera	shoots		gb,(P)	(15)	coastal	W
Ammophila arenaria	shoots	−8.1	gb	113,(70)	coastal dunes	1 A,W
Hordeae						
Agropyron junceiforme	shoots		gb	(23)	coastal	W
Agropyron pungens	shoots		gb	(80)	coastal	W
Triticum vulgare	shoots	−18.1	gb	64	grown in 100 mmol NaCl	1 D
Elymus arenaria	shoots		gb	(77)	coastal	W
Hordeum vulgare	shoots	−15 −17	gb	30 - 80	grown in 150 mmol NaCl	1 D
Aveneae						
Avena sativa	shoots	−17	gb	18	grown in 100 mmol NaCl	1 D
Danthonia caespitosa	shoots	−27	gb		saline	2 B
Chlorideae						
Chloris acicularis	shoots	−37	gb	85	halophyte	2 B
Chloris gayana	shoots		gb	4	grown in 150 mmol NaCl	1 D
Spartina anglica	shoots		gb,dsp,(P)	(120)	coastal	W
Spartina townsendii	shoots	−25	gb,dsp,(P)	258	salt marsh	1 A
Eragrosteae						
Diplachne fusca	shoots		gb	40	saline gravel	1 U
Lepureae						
Lepturus repens	shoots	−8.5	gb		arid coral island	2 E
Sporoboleae						
Sporobolus virginicus	shoots	−20	gb	101	salt marsh	2 B
Paniceae						
Spinifex hirsutus	shoots	−12	gb		coastal sand dune	2 F
Maydeae						
Zea mays	shoots	−13	gb	?	grown in 100 mmol NaCl	1 D
Zoysieae						
Zoysia macrantha	shoots	−15.5	gb	26	salt marsh	2 B
Uncertain						

Amphipogon caricinus	shoots	−22	gb		saline	2 B
Stenotaphrum secundatum	shoots	−13	gb	75	salt marsh	2 B
Triodia irritans	shoots	−28	gb	61	arid	2 B
Order Liliales						
FAMILY LILIACEAE						
Asphodelus fistulosus	shoots	−14.5	ND		saline	2 B
Dianella revoluta	shoots	−14	trig		arid sandy	2 B
Order Cyperales						
FAMILY CYPERACEAE						
Carex arenaria	shoots		ND		coastal	W
Scirpus maritimus	shoots		ND		coastal	W
Order Juncales						
FAMILY JUNCACEAE						
Juncus gerardii	shoots		ND		coastal	W
Juncus maritimus	shoots		ND		coastal	W
Order Aponogetonales						
FAMILY ZOSTERACEAE						
Zostera marina	shoots	−26	ND,(P)			2 Z
Order Juncaginales						
FAMILY JUNCAGINACEAE						
Triglochin maritima	shoots	−23	ND,(P)		salt marsh	1 A,W
Posidonia sp	leaves	−26	dsp			2 Z

[a]Classification according to Hutchinson (1973).

Table II continued

Abbreviations

1. Thin layer photodensitometry
2. Thin layer chromatography and periodide colorimetry
3. Ammonium reineckate or phosphotungstic acid precipitation
4. Chromatography and melting point determination
5. Periodide precipitation
6. Spectroscopy e.g. UV, IR, NMR or Mass

ND Not detectable
gb Glycinebetaine
trig Trigonelline
D+ Unknown Dragendorff positive
βab β-alaninebetaine
pb Prolinebetaine
OH-pb Hydroxyprolinebetaine
dsp Dimethylsulphoniopropionate
(P) Significant proline levels found (>15 μmol.g fresh wt^{-1})
tmkh 2-trimethylamino-6-ketoheptanoate

A. Storey et al. (1977)
B. Storey (unpublished data)
C. Storey (1976)
D. Storey and Wyn Jones (1977)
E. Pitman and Storey (unpublished data)
F. Condon and Storey (unpublished data)
G. Delaveau et al. (1973)
H. Cromwell and Rennie (1953)
I. Dasgupta et al. (1968)
J. Stanek and Domin (1909)
K. Rustembekova et al. (1973)
L. Susplugas et al. (1969)
M. Karawya et al. (1971)
N. Kapoor and Singh (1966).
O. Pulatova (1969)
P. Proskurnina and Utkin (1960)
Q. Paudler and Wagner (1963)
R. Larher and Hamelin (1975a)
S. Larher and Hamelin (1975b)
T. Baveja and Singla (1969)
U. Sandhu et al. (1981)
V. Comforth and Henry (1952)
W. Stewart et al. (1979)
X. Flowers and Hall (1978)
Y. Larher (1976)
Z. Wyn Jones unpublished data

of at least two families, the Labiatae and the Capparaceae. The hydroxyprolinebetaines are also found in these families. Prolinebetaine has also been reported in *Chrysanthemum* (Compositae), various *Citrus* (Rutaceae) and alfalfa (*Medicago sativa*, Leguminosae) (see Guggenheim, 1958, for early references). It is difficult with the evidence available to associate the prolinebetaine-accumulating families with particular ecological habitats. Some members of the Capparidaceae, for example *Capparis spp.*, are found in hot arid regions (Walter, 1971) and indeed, Comforth and Henry (1952) originally investigated *Capparis lomentosa* because of its reputed toxicity to camels! However, this cannot be said for the Labiatae, many of which, for example *Stachys spp.*, occur in temperate countries.

The distribution of quaternary ammonium compounds in the Capparidaceae (Capparaceae), 39 species from 20 genera, have been studied by McLean (1976), who found prolinebetaine and/or hydroxyprolinebetaine in all but 4 genera. Surprisingly, while 12 genera contained choline salts 5 contained 3-hydroxy-1,1-dimethyl pyrrolidinium salts. Trace quantities of glycinebetaine were found in some species.

C. β-ALANINEBETAINE

Although β-alaninebetaine was originally identified by Engeland (1909), it was only detected in plants in 1973, in the marine alga *Monostroma nitidium* (Abe & Kaneda, 1973). More recently it has been found in a higher plant, *Limonium vulgare* (Larher & Hamelin, 1975a). In this plant β-alaninebetaine is a major nitrogenous component and may exceed 100 μmol.g dry wt^{-1} (Larher, 1976). A second ammonio compound has been found in *Limonium vulgare* — 2-trimethylamino-6-ketoheptanoate (Table I) (Larher & Hamelin, 1975b). This compound was quantitatively far less important than β-alaninebetaine and occurred exclusively as the choline ester. Larher and Hamelin also reported that, in part, β-alaninebetaine occurs *in vivo* as the choline ester. As such, it would not be dipolar but would be a polycation with fixed positive charges at either end of the carbon ester chain.

So far only xerohalophytic members of the Plumbaginaceae have been reported to accumulate β-alaninebetaine. However, it remains to be seen if this rather specific distribution in higher plants is confirmed.

D. TRIGONELLINE AND HOMARINE

In a study of more than 100 plant species Klein et al. (1931) found trigonelline to be widely distributed in many families, including the Leguminosae, Solanaceae, Cruciferae, Compositae and the Graminae. In many cases it occurs in the presence of a second, quantitatively more

important betaine. However, unlike glycinebetaine, it is not accumulated to high levels in vegetative tissues, although Blaim (1962) found concentrations of greater than 80 μmol.g dry wt^{-1} in the seeds of a number of legumes. The isomer homarine has not been reported in higher plants, although it has been found quite widely in marine invertebrates, particularly the Crustaceae (Beers, 1967).

E. OTHER BETAINES

Tryptophanbetaine (Table I) has been found in the seeds of *Erythrina hypathorus* (Leguminosae), while histidinebetaine has been isolated from the fungi *Agaricus campestris* and *Boletus edulis* (for references see Guggenheim, 1958). Other betaine-like derivatives such as 2-mercaptohistidinebetaine, usually called ergothionein, have been found in fungi (Table I). This is similar to the simpler alkaloids and, indeed, in some texts the betaines are considered as alkaloids. These compounds, including histidinebetaine, etc., have also been reported in marine invertebrates (Ackerman & List, 1958).

F. RELATED AMMONIO COMPOUNDS

Choline, the alcohol related to glycinebetaine, is ubiquitously found in plants (Klein & Zeller, 1930; Toyosawa & Nishimoto, 1967; Storey & Wyn Jones, 1977) because of its role as a precursor of the major membrane constituent, phosphatidylcholine (lecithin). The sulphate and phosphate esters of choline have also been found (Table III). Tolbert and Wiebe (1955) identified choline-O-phosphate as a major phosphatic compound in the sap of tomato and barley, possibly involved in phosphate transport. Later, Nissen and Benson (1961) found that choline-O-sulphate accounted for 5–15% of labelled sulphate in *Zea mays*, *Hordeum vulgare* and *Helianthus annuus*. More relevant is the observation by Benson and Atkinson (1968) that, of the salt-secreting mangroves, *Avicennia* and *Aegialitis* spp. formed choline-O-sulphate, while *Aegiceras corniculatum* and *Acanthus ilicifolius* formed choline-O-phosphate in large quantities. They suggested that these compounds could be involved in salt transport.

Trimethylamine has been found in flowers, and Cromwell (1950) observed that the surface glands on the stems, leaves and perianth of *Chenopodium vulvaria* produced this amine, which probably arose from choline degradation. The trimethylamine oxide is widely found in fish and marine invertebrates (Prosser, 1973) and is important as an osmoticum.

Structurally related to these compounds is the plant growth regulator chlorocholine chloride (Table III). Various reports have suggested that its

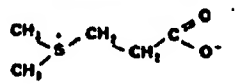
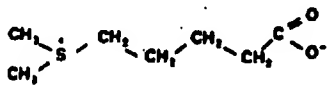
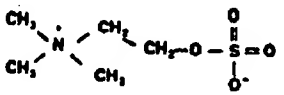
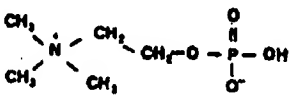
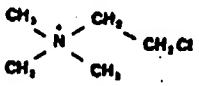
application increases the salt (El Damaty et al., 1964) and drought (Halevy & Kessler, 1963; Larher et al., 1965) tolerance of plants, but these have been disputed (cf. Robertson & Greenway, 1973; Imbamba, 1973).

G. RELATED SULPHONIO COMPOUNDS

The sulphonio analogues appear to be less common and less various than their ammonio counterparts; nevertheless, they have rather similar distributions. Challenger and Simpson (1948) isolated β-dimethylsulphoniopropionate from a marine alga and later (see Challenger, 1959) found that this compound occurs quite widely in marine algae, for example, *Polysiphonia* spp., *Enteromorpha* spp., and *Ulva lactuca*. It has also been reported in two fresh water species, *Oedogonium* sp. and *Ulothrix* sp., (Challenger, 1959). Recently it has been reported in a higher plant, *Spartina* sp. (Larher et al., 1977), and we have also found sulphonio compounds in large quantities in a

TABLE III

Name and structures of some methylated sulphonio and other related compounds.

Structures	Trivial names
	β-dimethylsulphoniopropionate (β-dimethylpropiothetin)
	δ-dimethylsulphoniopentanoate
	Choline-O-sulphate
	Choline-O-phosphate (Phosphoryl choline)
	Chlorocholine chloride CCC, Cycocel

marine angiosperm, *Posidonia* sp. (Wyn Jones & Hughes, unpublished data) and in *Wedelia biflora* (Gorham & Storey, unpublished data). Other, more complex sulphonio compounds occur in higher plants (the reader is referred to Challenger's book). In addition, Larher and Hamelin (1979) have isolated 5-dimethylsulphoniopentanoate from the flowers of *Diplotaxis tenuifolia* (Cruciferae).

III. TISSUE DISTRIBUTION OF BETAINES

A. VEGETATIVE TISSUES

Only in the case of glycinebetaine is extensive data on tissue distribution available, and most of our comments will be restricted to this compound. Both β-alaninebetaine (Larher, 1976) and glycinebetaine (Storey & Wyn Jones, 1977) are normally found in higher concentrations in shoot than in root tissues of mature plants. Cromwell and Rennie (1953) showed that the glycinebetaine content of *Beta vulgaris* leaves generally increases with age, reaching a maximum at flowering, but the root level declines. Indeed, in the young seedling, the root level exceeds that of the shoot. However, at any particular time the young, actively growing leaves contain much more glycinebetaine than mature, expanded leaves. These trends were confirmed in members of the Chenopodiaceae by Simenauer (1975), and by Stanek's original data (1916). Storey (unpublished data) also found levels of 30 μmol.g fresh wt^{-1} in unstressed *Atriplex spongiosa* apices compared with about 5 μmol.g fresh wt^{-1} in more mature tissue.

Recently, the distribution of glycinebetaine in barley throughout its life cycle has been studied by Ahmad (1978), and a similar pattern emerged. The less mature leaves have higher values, and these decrease as the plant age increases. There is some evidence of transfer from the flag leaf, which has high levels, to the developing grain, which also accumulates glycinebetaine as it matures.

B. NON-VEGETATIVE TISSUES

A number of papers have reported the occurrence of glycinebetaine, prolinebetaine (Cornforth & Henry, 1952) and trigonelline (Blaim, 1962) in seeds. For example, glycinebetaine has been reported in cotton seed (Pollock & Stevens, 1965), in quite remarkable quantity in beet seed (256 μmol.g fresh wt^{-1}), and in *Spartina x townsendii* seed (68 μmol.g fresh wt^{-1}) (Storey, 1976). Chittenden et al. (1978) found very high glycinebetaine concentrations in the mature wheat aleurone and embryo tissues but not in the starchy endosperm. The glycinebetaine content of the aleurone layer is much greater than the total internal amino acid pool. The amount of glycinebetaine did not change significantly during the first days of germination, but both it and the free amino acid pools were dramatically decreased by gibberellic acid treatment after 4 d of germination. Pearce et al. (1976) examined wheat ear tissues and, in Table IV, the glycinebetaine contents, as calculated from their data, are shown. As with the embryo and aleurone, partially dehydrated, but viable tissues, appear to have high levels.

TABLE IV
Glycinebetaine contents of wheat ear tissues.[a]

Tissue	Glycinebetaine (μmol.g dry wt^{-1})
anthers	96
palees	40
lemmas	27
glumes	19
rachis	14
grain	10
leaves	14

[a]Data calculated from Pearce et al. (1976).

IV. BIOSYNTHETIC PATHWAYS

A. GLYCINEBETAINE

Two pathways for the biosynthesis of glycinebetaine may readily be envisaged (Cromwell & Rennie, 1954a, 1954b; Delwiche & Bregoff, 1957): the sequential methylation of the parent amino acid, glycine, or the oxidation of choline, itself formed by the sequential methylation of ethanolamine. All the recent evidence favours the second pathway, and data showing incorporation of ^{14}C-choline into glycinebetaine have been obtained with wheat (Bowman & Rohringer, 1970), barley (Hanson & Nelson, 1978) and a number of chenopods (Cromwell & Rennie, 1954a; Delwiche & Bregoff, 1957) (Fig. 1). This is also the pathway prevalent in animals (Paxton & Mayr, 1962).

As noted by Hanson and Nelson (1978), the major precursors of glycinebetaine, glycine, serine and formate are all intermediates in the photorespiratory or photo-oxidative carbon cycle. No enzymological data are available for higher plants, and nothing is known of the presumed choline dehydrogenase (choline oxidase) or glycinebetaine-aldehyde dehydrogenase enzymes, despite attempts to isolate them from chenopod tissue (Speed, 1972). Hanson and Nelson (1978) pointed out that, during the sequential methylation steps, there is a net demand for reducing power, which, using formate as the donor, would approximate to an NADPH requirement four times the rate of glycinebetaine accumulation. It should also be noted that there is a net production of a proton per glycinebetaine accumulated (Fig. 1).

The catabolic pathway has not been studied in plants but, by analogy

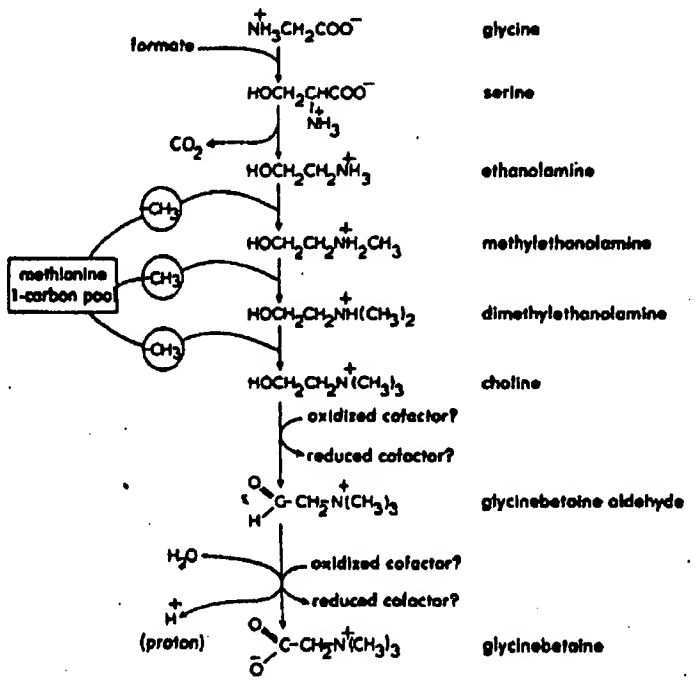


FIG. 1. Probable pathway for glycinebetaine biosynthesis.

with the microbial degradative pathway (Kortstee, 1970; Wyn Jones et al., 1973), the probable path is through the sequential demethylation of glycinebetaine to glycine.

B. PROLINEBETAINE

In their study of prolinebetaine biosynthesis in alfalfa seedlings, Wiehler and Marion (1958) found that 2-week-old plants could convert ornithine and glutamate to proline and, on the further addition of both methionine and folic acid, ^{14}C-methyl label from the methionine was isolated in prolinebetaine. Thus, a pathway involving the sequential methylation of proline was proposed. In a later paper Robertson and Marion (1960), while able to show the conversion of N-methylproline (hygric acid) to prolinebetaine, were unable to show the conversion of proline itself and expressed some reservations whether the simple pathway of sequential methylation was in fact correct.

While prolinebetaine is the major betaine in young alfalfa seedlings, trigonelline and pipecolatebetaine are equally important in seeds. Robertson and Marion (1959) made the interesting suggestion that the biosynthesis of these other betaines may only be initiated after anthesis.

C. β-ALANINEBETAINE

By feeding ^{3}H-β-alanine and ^{14}C-methyl-labelled methionine to *Limonium vulgare* leaves, Larher (1976) found that β-alaninebetaine was probably synthesized by the sequential methylation of the parent amino acid, and that methionine could well be acting as the methyl donor (see Fig. 2). Again the enzymology is unknown. In this context it is interesting to note Challenger's (1959) speculations on the relationship between the presence of dimethylsulphonio propionate and β-alanine in many marine algae (Ericson & Carlson, 1953). He suggested that the decomposition of the sulphonio compounds to dimethylsulphide ($(CH_3)_2S$), acrylic acid (CH_2=CHCOOH) and a proton (H^+), which has been shown to be enzyme mediated (Cantoni & Anderson, 1956), could initially liberate $^+CH_2CH_2COO^-$, which would yield β-alanine, with ammonia. However, we are aware of no recent biochemical work to confirm this speculative link between β-alanine, or its betaine, and dimethylsulphoniopropionate.

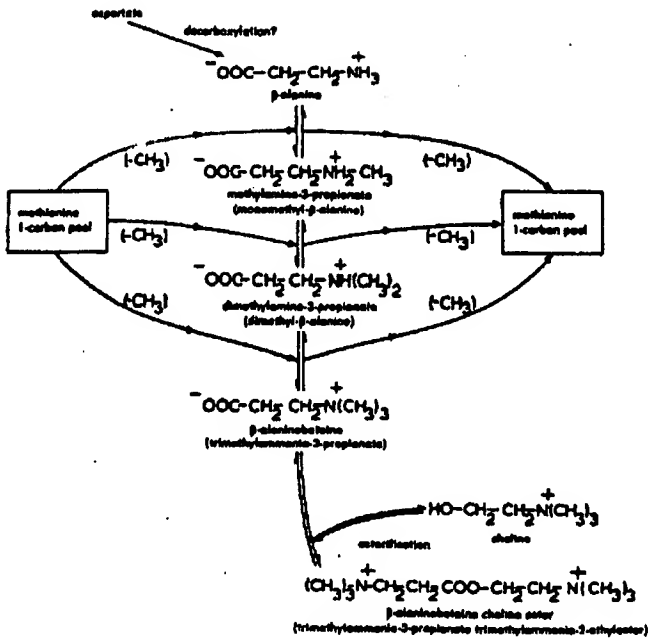


FIG. 2. Possible pathway for β-alaninebetaine biosynthesis.

V. ACCUMULATION OF BETAINES IN STRESSED TISSUES

A. GLYCINEBETAINE

Although there was an intermittent interest in the distribution and biosynthesis of glycinebetaine in higher plants, and a few papers appeared describing the effects of added glycinebetaine on leaf disc and coleoptile expansion (Wheeler, 1963, 1965, 1969), little progress was made in exploring its function. However, this situation is changing rapidly following the key observation that the accumulation of the compound could be induced by salt or water stress (Storey & Wyn Jones, 1975; Storey, 1976). Evidence for progressive glycinebetaine accumulation in plants exposed to increasing

external salt (NaCl) concentrations is now available for *Chloris gayana* (Storey & Wyn Jones, 1975), *Hordeum vulgare* cvs. *California Mariout* and *Arimar* (Wyn Jones & Storey, 1978a), *Diplachne fusca* (Sandhu et al., 1981), *Spartina x townsendii* (Storey & Wyn Jones, 1978b), *Atriplex spongiosa* and *Suaeda monoica* (Storey & Wyn Jones, 1979), and *Spinacia oleracea* (Coughlan & Wyn Jones, unpublished data).

Less complete but similar data are available for *Zea mays* var. *WF9 x M14*, *Triticum vulgare* var. *Capelle Desprez*, *Avena sativa* (Storey & Wyn Jones, 1977) and *Suaeda maritima* (Flowers & Hall, 1978), and there can be little doubt that external NaCl induces glycinebetaine accumulation in many, but perhaps not all members of the Gramineae and Chenopodiaceae (see *Puccinellia maritima* in Table II). However, the amounts accumulated vary from very small, in *Zea mays* (0.5–2 μmol.g fresh wt^{-1}), to very large, in some halophytic species (about 100 μmol.g fresh wt^{-1}), some of which have very high basal levels even in the absence of external salinity, for example *Suaeda monoica* with about 50 μmol . g fresh wt^{-1} (Storey & Wyn Jones, 1975). In *Atriplex spongiosa* (Storey & Pitman, unpublished data) other salts, such as KCl, Na_2SO_4 and $MgCl_2$, also bring about glycinebetaine accumulation. However, glycinebetaine accumulation does not occur in salt-stressed *Atriplex spongiosa* plants when the relative humidity is maintained at 95–100%; these growth conditions do not result in the net salt accumulation in the leaves observed at lower relative humidities (Storey & Pitman, unpublished data).

There is less evidence to show that water stress brings about an increase in the glycinebetaine content of plants. Nevertheless, polyethylene glycol treatment and water withdrawal induces glycinebetaine accumulation in barley cultivars (Fig. 3; Hanson & Nelson, 1978), and similar observations have been made with *Spinacia oleracea* (Coughlan & Wyn Jones, 1980) and *Atriplex spongiosa* (Storey & Pitman, unpublished data). Thus, it is highly likely that accumulation can be brought about by both salt and water stress, although the former appears to be more effective. This may be due to the substantially greater osmotic pressure change in salt-stressed compared with water-stressed tissue. However, in marked contrast to proline accumulation (cf. Hsiao, 1973), glycinebetaine accumulation cannot be regarded as a general plant response to water stress, as many species, for example *Lycopersicon esculentum*, *Pisum sativa*, *Daucus carota* (Storey & Wyn Jones, 1977), do not react in this manner. This difference is underlined by the phytotaxonomic data in Table II; glycinebetaine has not been detected in all plants having high sap osmotic pressures found in saline environments, or xeric environments.

Little is known of the biochemical mechanisms leading to the induction or activation of the glycinebetaine biosynthetic pathway. However it is

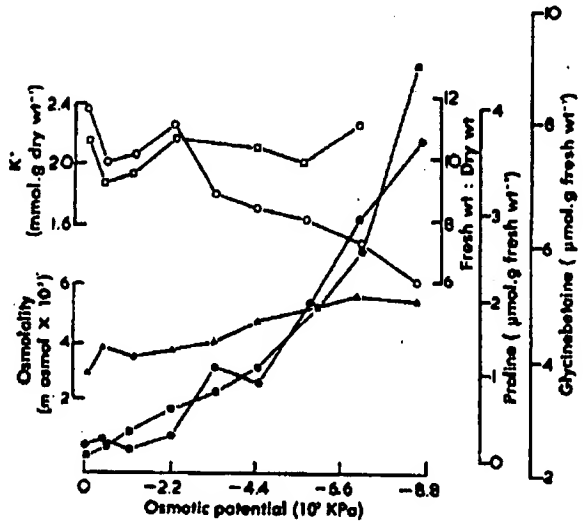


FIG. 1. Influence of water (polyethylene glycol) stress on glycinebetaine and proline levels and related parameters in barley, cv. California Mariout, subjected to a gradual incremental stress. (O) fresh wt: dry wt ratio; (□) K^+; (▲) osmolality; (■) glycinebetaine; (●) proline. (From Wyn Jones and Storey, 1978a.)

interesting to note that Lawlor and Fock (1978) found that the rate of photorespiration relative to photosynthesis increased in maize following water stress. Further, a greater proportion of ^{14}C from labelled CO_2 was found in the amino acids of the photorespiratory pathway, glycine and serine, both probable precursors of glycinebetaine (Fig. 1).

B. COMPARISON OF GLYCINEBETAINE AND PROLINE ACCUMULATION AND CONSUMPTION

The differences and similarities between glycinebetaine and proline accumulation in stressed plant tissues deserve further consideration. Hanson and Nelson (1978) and Wyn Jones and Storey (1978a) noted the close relation between the levels of these two solutes in barley subjected to water or salt stress. However, unstressed glycinebetaine levels are ten-fold higher than those of proline. Since a gradual stress induces a similar rate of glycinebetaine

and proline accumulation (about 0.1–0.5 µmol . g fresh $wt^{-1}.d^{-1}.25\ mM^{-1}$ NaCl increment: Wyn Jones & Storey, 1978b), the proportional increase in proline is greater, although the former is still quantitatively more significant. In conditions of salt or osmotic shock such as employed by Hanson and Nelson (1978) and in some experiments by Wyn Jones and Storey (1978a), the proportional increase in proline content is even greater, so that final proline levels exceed those of glycinebetaine. Thus, in barley, there appears to be a relationship between the relative rates of proline and glycinebetaine accumulation and the method of stress application (see also Coughlan & Wyn Jones, 1980).

It is probable that there are differences in the rates of induction of proline and glycinebetaine in that glycinebetaine increases have only been recorded after 24 h whereas proline accumulation has been recorded in tens of minutes (Singh et al., 1973). However, this is not borne out in the data of Hanson and Nelson (1978), and further experimentation is required.

A major difference between the two solutes lies in their rates of degradation. The data of Bowman and Rohringer (1970) on wheat, Storey (1976) on maize, and Hanson and Nelson (1978) on barley indicate that the rate of glycinebetaine degradation in plant tissue is slow in contrast to proline (Singh et al., 1973). A direct comparison is shown in Figure 4 in an experiment in which both NaCl and polyethyleneglycol stress were applied and then withdrawn. While proline levels decreased rapidly after stress withdrawal, particularly in polyethyleneglycol-treated tissues, glycinebetaine remained almost constant. The relative inertness of glycinebetaine led Hanson and Nelson (1978) to suggest that it might be employed as a cumulative index of water stress in barley.

The differences between the patterns of glycinebetaine and proline accumulation are quite pronounced in the salt tolerant chenopods, *Atriplex spongiosa* and *Suaeda monoica* (Storey & Wyn Jones, 1978b). In these species, glycinebetaine increases, particularly on a dry-weight basis at low salinities, which stimulate both the growth of the plants and their succulence. Proline accumulation occurs either as a transient response to the initial salt shock of low salinities or as a long-term effect in plants exposed to very high, inhibitory salt levels. Thus, glycinebetaine is strongly correlated with sap osmotic pressure (*A. spongiosa*, $R = 0.95$, $p < 0.01$; *S. monoica*, $r = 0.98$; $p < 0.01$), whereas proline accumulation mirrors the decrease in yield and fall in tissue water content at high salinities. In barley, even fairly low salinities cause some growth inhibition and a decline in fresh weight to dry weight ratio (Storey & Wyn Jones, 1978a). Thus, the differences between the behaviour of glycinebetaine and proline in these species may be due to their different growth and morphological responses to salt (NaCl), and the absence of a significant succulence and growth response in barley.

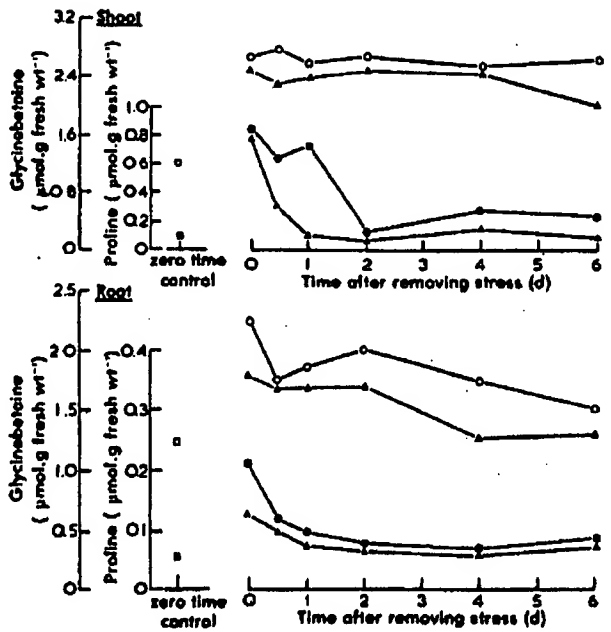


FIG. 4. Comparison of glycinebetaine and proline levels in barley cv. Arisner subject to, and then released from, NaCl and water (polyethylene glycol) stress. Open symbols, glycinebetaine (□, △, ○) subjected to salt (○) and polyethylene glycol (△) stress. Closed symbols, proline (■, ●, ▲), subjected to salt (●) and polyethylene glycol (▲) stress. The final stress, −0.8 MPa, was applied incrementally over 36 h and maintained for 24 h before being removed at day 0. (Ahmad and Wyn Jones, unpublished data.)

C. β-ALANINEBETAINE AND PROLINEBETAINE

In *Limonium vulgare*, Larher (1976) found that the shoot levels of β-alaninebetaine increase with external salinity (Table V) to a final concentration (over 100 μmol . g dry wt^{-1}), fairly similar to the glycinebetaine levels (highest recorded levels over 400 μmol . g dry wt^{-1}). In this species, also, there is a greater proportional increase in proline content although, at the highest salinity, β-alaninebetaine is still, quantitatively, the more important (Table V). Unfortunately, no growth or inorganic ion data were included so

that detailed comparisons with the members of the Chenopodiaceae are precluded.

Whilst the presence of prolinebetaine in alfalfa has been known for many years (Steenbock, 1918), only recently (Wyn Jones & Owen, unpublished data) has it been demonstrated that this compound also accumulates under saline conditions (Table VI). In this work, two alfalfa cultivars of very different salt tolerances were compared, and both the basal and the induced levels of prolinebetaine were found to be much higher in the more tolerant cultivar.

TABLE V

Changes in some soluble nitrogen-containing compounds in young *Limonium vulgare* plants grown at different salinities.[a]

Compounds	Control medium[b]	167 mM NaCl	333 mM NaCl
	(μmol.g dry wt^{-1})		
proline	trace	10.7	65.4
α-alanine	17.7	20.0	19.3
glutamate	15.7	7.7	7.7
amides	72.9	24.7	18.7
γ-aminobutyrate	19.0	11.8	10.8
total free amino acids and amides	147	102	141
β-alaninebetaine	46.9	67.1	100.2

[a]Data from Larher (1976).
[b]Plants grown in Hoagland's medium with the addition of NaCl as indicated.

TABLE VI

Effect of salinity on the levels of prolinebetaine and related compounds in the shoots of alfalfa cv 'Hawassi' (2nd cut).[a]

Salinity level[b] (mM NaCl)	Sap osmolality (m osmol)	Proline-betaine	Proline	Choline	Trigonelline	Total onium compounds
		mM in sap				
0	590	12	3.6	1.6	>0.5	18
50	625	15	1.2	2.6	>0.5	18
100	813	17	2.7	2.3	>0.5	19
200	930	18	4.1	0.5	>0.5	23

[a]Unpublished data from Wyn Jones and Owen.
[b]Plants grown in Huagland's medium with added NaCl as shown.

VI. POSSIBLE PHYSIOLOGICAL FUNCTIONS

A. GLYCINEBETAINE AS A CYTOPLASMIC OSMOTICUM

Early studies (Cromwell & Rennie, 1954a, 1954b; Byerrum et al., 1955) suggested that glycinebetaine might be involved in methyl transfer, but in some cases (Sribney & Kirkwood, 1954) the glycinebetaine methyl group was not transferred readily to a range of acceptors. This work failed to explain the high concentrations found in some plants. However, the close correlation between glycinebetaine concentration and sap osmotic pressure in leaves of various species (Wyn Jones et al., 1977a, 1977b; Storey & Wyn Jones 1978b, 1979) indicated that the compound might have an osmotic role. Evidence of the sensitivity of some cytoplasmic enzymes and organelles from halophytic plants to high concentrations of inorganic ions (Flowers et al., 1977), and increasing evidence of ion compartmentation between cytoplasm and vacuole (see Jeschke, 1979; Wyn Jones et al., 1979), led a number of workers (e.g. Flowers, 1975; Osmond, 1976) to postulate a requirement for cytoplasmic non-toxic osmotica. Wyn Jones and his colleagues (1977a, 1977b) produced evidence that glycinebetaine might fulfil this role in some plants. They also suggested that the cytoplasm would be K^+-specific, with the majority of the Na^+ and Cl^- ions, if present, sequestered in the vacuole. Recently the possible free ion concentrations (activities) in the cytoplasm have been considered in some detail (Wyn Jones et al., 1979), and a unifying hypothesis involving the effects of ions on protein synthesis has been advanced.

If glycinetetaine is utilized as a cytoplasmic osmoticum when the cell sap osmotic pressure exceeds a basal level (about 0.8–1.0 MPa) (Wyn Jones et al., 1977a), then two basic requirements must be met: (i) the solute must be non-toxic to cytoplasmic functions; and (ii) the solute must be preferentially located in the cytoplasm as the concentrations observed can only make a major osmotic contribution if the solute is restricted to a fairly small percentage of the cell volume. (This requirement may also be related to the evidence for the preferential localization of Na^+ and Cl^- in the vacuole: see Jeschke, 1979.)

It may also be suggested that the cytoplasmic osmoticum must be highly water soluble and not carry a net charge which could affect the charge balance of the cytoplasm. Glycinebetaine is dipolar (zwitterionic) and, unlike amino acids, has only one titratable group, the carboxyl, which has a pKa of 1.83 at 25°C, as the cationic charge on the nitrogen is fixed. Thus, over the physiological pH range, glycinebetaine will have no net charge and no buffering capacity. The internal charge compensation is such that it will pass through a mixed bed ion exchange column at neutral pHs (Carruthers et al., 1960). Glycinebetaine is extremely water soluble (157 g.100 ml^{-1} at 19°C), but, effectively, is insoluble in ether (Dawson et al., 1969).

Evidence that glycinebetaine, in concentrations up to 0.5–1.0 x 10^3 mM, did not inhibit malate dehydrogenase (decarboxylating) was presented by Wyn Jones et al. (1977b) and confirmed by Flowers et al. (1978). A more detailed study (Pollard & Wyn Jones, 1979) has shown that a number of plant and animal enzymes are not inhibited by glycinebetaine and that the NaCl inhibition of some enzymes is partially relieved by glycinebetaine (200–500 mM). The partial protection probably is not mediated by direct binding of glycinebetaine to the enzyme (cf. Schobert, 1977). We have also found that active intact mitochondria from wheat embryo (Pollard & Wyn Jones, unpublished data), and Type A chloroplasts from spinach leaves (Larkum & Wyn Jones, 1979) may be isolated and studied in glycinebetaine as an alternative osmoticum to sucrose, mannitol or sorbitol. Polysome stability is unimpaired by 500 mM concentrations (Brady & Wyn Jones, unpublished data), while *in vitro* protein synthesis using the wheat embryo system is not significantly inhibited by 250–300 mM glycinebetaine over and above a basal 120 mM potassium acetate medium (Speirs, Brady & Wyn Jones, unpublished data). Thus, the non-toxicity of glycinebetaine is firmly established and the first condition for its role as a cytoplasmic osmoticum has been satisfied.

The second criterion, namely conclusive evidence for a preferential, but not necessarily exclusive, cytoplasmic localization, is more difficult to demonstrate. Nevertheless, three lines of evidence point in this direction.

1. Analyses of tissues of low vacuolation, for example *Atriplex spongiosa* apices, wheat embryo or aleurone, showed very high glycinebetaine concentrations, whereas vacuolated, older tissues have lower levels (see Section III).
2. By a histochemical technique, Hall et al. (1978) showed glycine betaine to be exclusively located in the cytoplasm of *Suaeda maritima* leaf cells.
3. Employing the technique of whole vacuole isolation developed by Leigh and Branton (1976) using red beet storage tissue, Leigh, Ahmad and Wyn Jones (unpublished data; see also Wyn Jones et al., 1977a) found glycinebetaine to be partially excluded from vacuoles.

All the individual techniques may be criticized, but taken together, the results support the original hypothesis and make a strong case for glycinebetaine being largely cytoplasmic. This hypothesis may be applied to halophytes and certain xerophytes, but it is not relevant to ephemeral or succulent xerophytes which maintain low sap osmotic pressure (Walter, 1971). In one sense the concept is more applicable to salt-accumulating halophytes. This is because glycinebetaine, in contrast to proline, is very slowly metabolized in plants and is a semi-permanent end product of metabolism. There is evidence that Na^+ is also held relatively immobile in vacuoles of some plants and is not transported rapidly out of leaves (Yeo,

1976; Jeschke, 1979), so that the accumulation of the metabolically inert glycinebetaine in the cytoplasm may be considered as balancing the absorption of rather immobile Na^+ salts into the vacuoles. In this context, there is unfortunately a lack of good evidence on the distribution of Cl^- (Wyn Jones et al., 1979), but it may also be vacuolar. In the Chenopodiaceae, where this model may be applied best, the major balancing vacuolar anion may be oxalate (Osmond, 1968). Many of these Chenopodiaceae are xerohalophytes and are found in dry, saline habitats. There is a clear relationship in many cases between salt and water stress tolerance.

B. OTHER ASPECTS OF THE PHYSIOLOGICAL CHEMISTRY OF GLYCINEBETAINE

Glycinebetaine has been presented as a solute notable for its lack of toxicity and intracellular metabolic stability. It is, however, rapidly degraded extracellularly by soil micro-organisms (Kortstee, 1970; Wyn Jones et al., 1973). Early work on the addition of glycinebetaine to root media was hampered by microbial interference, but, by using axenic cultures, the positive effects of this compound were demonstrated. Changes in the shoot K^+ and Na^+ contents of treated plants have also been observed (Storey, 1976; Ahmad, 1978). Recently, the possible influence of external glycinebetaine and glycinebetaine loading of tissues on ion fluxes has been studied in greater detail (Ahmad, 1978; Ahmad & Wyn Jones, 1978). Perhaps the most interesting of the phenomena observed was the influence of glycinebetaine loading of barley roots on tonoplast fluxes (Ahmad, Jeschke & Wyn Jones, unpublished data). Using a technique which allowed the separate determination of ^{22}Na efflux and transport in excised barley roots, glycinebetaine loading, or a metabolic consequence thereof, was found to increase Na^+ flux from the cytoplasm to the vacuole and to increase the vacuolar Na^+ concentration. This observation implies a link between the accumulation of the cytoplasmic osmoticum and the partitioning of Na^+ between cytoplasm and vacuole (Table VII). It further suggests, not surprisingly, that the passive model of cytoplasmic osmotic regulation may be superseded by a dynamic model, as various feed-back controls are clearly required between cytoplasm and vacuole to maintain a uniform water potential across the tonoplast. One should also note the structural similarity of glycinebetaine and other *onium* compounds found in nature to compounds well known to modify membrane behaviour (Fluck & Jaffe, 1974; Davidson, 1976).

It is not difficult to relate suggestions that glycinebetaine may have a role in frost resistance (Bokarev & Ivanova, 1971; Sakai & Yoshida, 1968) to the evidence presented above. Similarly, Rafaeli-Eshkol (1968) and Rafaeli-Eshkol and Avi-Dor (1968) originally reported that choline, probably after

TABLE VII
Influence of glycinebetaine loading of excised barley roots on ^{22}Na fluxes. Roots loaded in presence of 1.0 mM glycinebetaine for 24 h.[a]

Tissue	Plasmalemma influx	Plasmalemma efflux	Tonoplast influx	Vacuole content
	μmol. g fresh $wt^{-1}.h^{-1}$			μmol. g fresh wt^{-1}
Glycinebetaine				
not loaded	0.65	0.41	0.047	23.0
loaded	0.70	0.47	0.082	30.1

[a]Data from Ahmad, Jeschke and Wyn Jones (unpublished).

conversion to glycinebetaine, increased the salt tolerance of a moderately tolerant bacteria Ba_1. More recently, Shkedy-Vinkler and Avi-Dor (1975) have shown the importance of glycinebetaine in protecting a membrane function (respiration) in bacteria exposed to NaCl. They did not stress an osmotic role for glycinebetaine but their data showed a salt-promoted uptake of the solute and an intracellular glycinebetaine concentration of 800 mM under the high salt condition. Thus, it is possible that the solute has an osmotic role as well as a membrane modifying function. These two roles are not necessarily incompatible.

C. POSSIBLE FUNCTION OF β-ALANINEBETAINE

Little is known of the metabolic function of β-alaninebetaine. While the leaf content of β-alaninebetaine increased with external salinity (Larher, 1976), interpretation is complicated by a significant, but ill-defined, proportion being present as the choline ester. As a choline ester this compound, and the 2-trimethylamino-6-ketoheptanoate, would have two fixed cationic charges and would require balancing anions. In contrast to the evidence for glycinebetaine, Larher (1976) observed, by a cytochemical technique, that β-alaninebetaine and/or its choline ester, were sited in the vacuole. If this is confirmed, the rather lower levels found would exclude any simple osmotic role. Drawing an analogy with the structures of acetylcholine antagonists, Larher tentatively suggested that the β-alaninebetaine choline ester may have a role in controlling the permeability of membranes to Na^+ and Cl^-. Although Larher's work did not include inorganic ion analyses, Mg^{2+} might be added to these ions as other data show the Plumbaginaceae to be very rich in this ion (Storey et al., 1977). A further factor worth considering in future work may be a similarity of these compounds to the polyamines, for example spermidine (Basso & Smith, 1974).

VII. CONCLUDING DISCUSSION

A compound which accumulates in a plant under conditions of water or salt stress may be a by-product, possibly toxic, of that stress, or a possible adaptation to help withstand the effects of the stress. Hanson and Nelson (1978) take the view that the only interpretation of glycinebetaine accumulation justified by the data is the former, and they see both glycinebetaine and proline accumulation as a method for consuming excess reducing power. However, from a consideration of its taxonomic distribution and the glycinebetaine responses of salt tolerant members of the Chenopodiaceae, we take the view that glycinebetaine accumulation is an adaptive response to salt and, possibly, water stress. This view is reinforced by an examination of glycinebetaine distribution in other species, such as marine invertebrates, where the compound appears to be accumulated as an intracellular osmoticum. Further, circumstantial support comes from the comparative data on the effects of inorganic ions and glycinebetaine on enzymes, ribosomes, mitochondria and chloroplasts, which show that glycinebetaine, but not the inorganic ions, is tolerated at high concentrations.

It does not follow from this analysis that the rate or degree of glycinebetaine accumulation is a measure of tolerance (see Wyn Jones & Storey, 1978b). Many physiological and biochemical factors are involved in salt and drought tolerance, and the ability to accumulate glycinebetaine, albeit as an important cytoplasmic osmoticum, may not be limiting. While this solute appears to be the preferred osmoticum in some species, other species have evolved other mechanisms and, in all probability, other cytoplasmic osmotica, of which proline may be an example. There is therefore, *a priori*, no conflict between the hypotheses regarding the two solutes. Nevertheless, some differences exist between the two, particularly in their rapidity of accumulation and metabolic lability, which suggest that proline accumulation could be the major response to a transient stress or shock, and glycinebetaine to a longer, possibly more gradual, stress usually involving net salt accumulation.

We have suggested that the concept of a cytoplasmic non-toxic solute is applicable to seeds and other partially dehydrated tissues such as anthers, as well as xerophytic and halophytic plants. These, indeed, may be examples in which the accumulation of the more metabolically inert solute is favoured. It will be apparent from Section II that a wide range of betaines have been found in seeds. Whether they all fulfil an osmotic-protective role similar to that proposed for glycinebetaine remains to be seen, but their structures and the evidence on the role of β-alanine choline ester makes such a simple explanation unlikely.

CHAPTER 10

Proline Accumulation: Physiological Aspects

D. ASPINALL AND L. G. PALEG

PHYSIOLOGY AND BIOCHEMISTRY OF DROUGHT RESISTANCE IN PLANTS

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Drought and salt tolerance: towards understanding and application

Kent F. McCue and Andrew D. Hanson

Only a few desirable traits in plants are understood at the molecular level. Fewer still are amenable to manipulation by molecular biological techniques currently available. The accumulation of glycine betaine, an osmoprotectant compound implicated in drought and salt tolerance, may be one such trait.

By far the most important factors limiting crop productivity are environmental stresses, of which lack of water (drought) is the most serious[1]. Salinity is also a major constraint; this is a significant problem in the USA because it affects some of the otherwise most productive agricultural areas, including almost 30% of California's irrigated land[2]. Increased drought- and salt-tolerance have therefore been major objectives of plant breeding programs for regions where rainfall or irrigation supply is limiting, where soil salinity is high, or where water quality is poor. Although conventional breeding methods have had some encouraging successes, overall progress has been slow[1]. This has focused interest on the possibilities of genetic engineering for drought- and salt-tolerance[3]. Is this interest justified? We think it is, provided that it is tempered by an understanding of the inherent limitations of the genetic-engineering approach.

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Adaptations of plants to stress

Plants have evolved many types of adaptations to drought and salinity which can be classified as belonging to one of four levels. The most complex mechanisms, requiring the interplay of many gene products, are at the developmental level; the simplest, perhaps involving only a single gene product, at the biochemical level (Fig. 1). Unfortunately, with the current levels of knowledge of plant genetics and metabolism, genetic engineering can only be applied to biochemically definable traits; desirable but complex traits remain out of reach. Such complex traits include water-use efficiency (the amount of dry matter or harvestable yield produced per unit of water used)[4], root morphology and anatomy[5], and the ability to exclude salt while maintaining water flow through the plant[6]. While biochemical adaptations do lie within reach of genetic engineering, our understanding of integrative processes in plants is not yet adequate for predicting whether alteration of these traits will translate into beneficial effects at the crop level.

Osmoprotectants in plants

What kinds of biochemical stress-resistance traits are sufficiently defined for genetic engineering? Here we present the case in favor of glycine betaine accumulation, beginning with some background on the physiology of drought and salt stress. A problem for all living organisms in dry or saline environments is to maintain water content; this is achieved by solute accumulation, which lowers solute potential. The solutes accumulated in the cytoplasm must be non-toxic ('compatible') with respect to metabolic processes; that is, they should not interfere with protein structure or function when present at high concentrations[7]. Only a few types of organic compounds (osmoprotectants) meet this requirement; quaternary ammonium compounds such as glycine betaine are among the commonest of these, and occur in bacteria, cyanobacteria, algae, higher plants and animals[8]. Other osmoprotectants found in diverse organisms include various polyols, the amino acid proline, and tertiary sulfonium compounds. Many other solutes, especially inorganic ions such as Na^+, Cl^-

and SO_4^{2-}, are quite toxic. For salinity stress, where the cytoplasm may be exposed to elevated levels of toxic ions, protection of protein function in the presence of such ions ('haloprotection') is desirable. Studies with cyanobacteria have shown that of several compatible solutes examined, only glycine betaine provided haloprotection, relieving the inhibitory effects of high salt levels on enzyme activity[9].

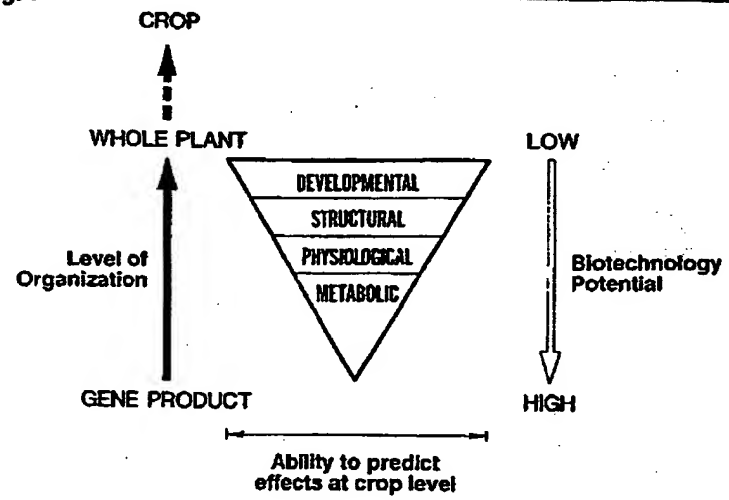
Figure 2 shows the chemical structures of glycine betaine and some other osmoprotectants found to accumulate in plants, and the legend gives information on their taxonomic distribution. Glycine betaine accumulation is quite widespread, occurring in species from many large plant families such as the Asteraceae, Chenopodiaceae, Poaceae and Solanaceae[10]. Interestingly, glycine betaine does not accumulate in many important crop species such as tomato, potato and rice, all of which have relatives which are accumulators. This makes such species good targets for genetic engineering of the glycine betaine pathway. Some of the less common osmoprotectants shown in Fig. 2 may also become of interest for genetic engineering as more is learned about them.

A very important point about the accumulation of glycine betaine (and probably other osmoprotectants) in higher plants is that this accumulation is restricted largely to the cytoplasmic compartments of the cells (Fig. 3a). The compartmentation of glycine betaine depends, presumably, upon specific transport mechanisms, but very little is known about betaine transport systems in plants, and it is not clear whether or not such transport systems are specific to betaine-accumulating species.

Glycine betaine biosynthesis

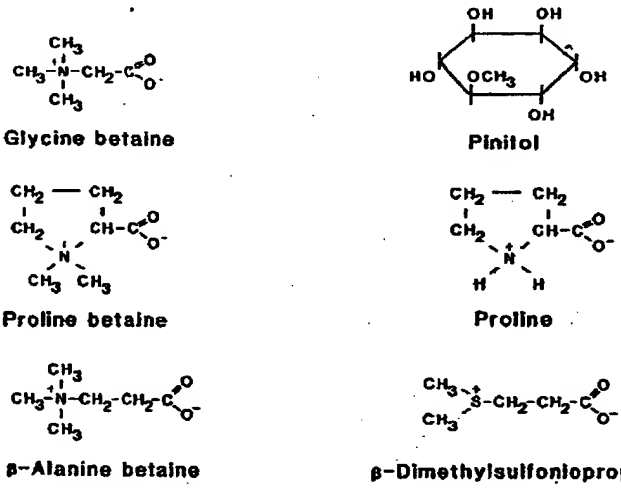
The synthesis of glycine betaine requires only two enzymes in a two-step oxidation of choline (Fig. 4). The first enzyme, choline monooxygenase (CMO), has been partially purified and characterized from spinach[24]. CMO is localized in the chloroplast stroma; it is stimulated by Mg^{2+} and has a pH optimum of ~8. The reaction catalysed by CMO requires molecular oxygen and reduced ferredoxin[24]. The second enzyme is betaine aldehyde dehydrogenase (BADH). The majority

Fig. 1



Some relationships between stress resistance and biotechnology. Crop stress resistance can be viewed as the sum of traits expressed at four levels of organization. At the highest level are developmental traits (e.g. time of flowering). Below this come structural traits (e.g. rooting patterns or leaf waxiness). The next lower level comprises physiological traits such as water-use efficiency. At the lowest organizational level are metabolic or biochemical resistance traits; since these traits can be construed in terms of small numbers of enzymes (gene products), they are potential targets for biotechnology, though it is difficult to predict the impact on crop yield of intervening at the biochemical level. The organizational levels are framed by a triangle whose width is proportional to our ability to predict the effects of genetic manipulation on stress resistance at whole-plant and crop levels; for example, intervention at the developmental level resulting in an earlier/later time of flowering will predictably alter stress resistance, whereas the effects on resistance of altering metabolic traits, such as betaine accumulation, are harder to predict.

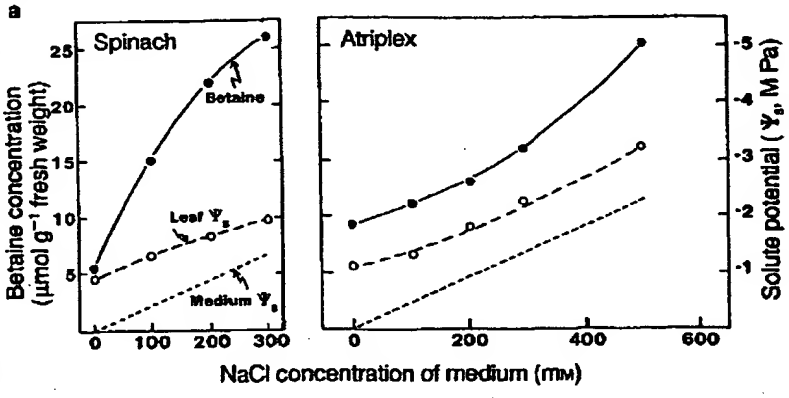
Fig. 2



Some compatible solutes found to accumulate in higher plants. Certain of these occur very widely, such as proline. Glycine betaine accumulation occurs in several large and important families including the Asteraceae, Chenopodiaceae and Poaceae. Other betaines have a more limited distribution, such as proline betaine found in the Fabaceae and the Lamiaceae, and β-alanine betaine in the Plumbaginaceae. Other compatible solutes are also limited in occurrence, such as D-pinitol found primarily in the Fabaceae and some Caryophyllaceae, and β-dimethylsulfoniopropionate, found in the marine grass Spartina. (Data from Refs 10–13.)

Fig. 3

a



b *Solutes in a salinized chenopod leaf cell*

	K^+	Na^+	Cl^-	Organic acids	Betaine
	(mM)				
Vacuole	150	200	150	100	<1
Cytosol and chloroplast	120	<50	<50	60	300

Glycine betaine as a compatible cytoplasmic solute in plant cells. (a) *Typical patterns of osmotic adjustment and betaine accumulation in leaves of salt-stressed plants of the family Chenopodiaceae. Spinach is representative of moderately salt-tolerant plants of this family, whereas* Atriplex spongiosa *is a more halophytic species. In both species, as salt is added to the growth medium, the leaves accumulate betaine and other solutes, maintaining their solute potential below that of the medium by a fairly constant 0.6 to 1 MPa. This differential provides the driving force for growth and water uptake.* (Data from Refs 14, 15.) (b) *The major solutes which accumulate in salinized leaves are Na^+, Cl^-, organic acids, and glycine betaine. Salinization causes relatively little change in vacuolar and cytoplasmic K^+ levels. Na^+ and Cl^- accumulate mainly in the vacuole, and betaine mainly in the cytoplasm (chloroplasts + cytosol). The vacuole occupies about 90% of the cell volume, and the chloroplast and cytosol about 5% each. Hence osmotic adjustment of the bulk of the cell water is achieved with Na^+ and Cl^-, which are readily available from the salinized growing medium, but are toxic to metabolism. Glycine betaine, which is non-toxic but energetically expensive, is used for osmotic adjustment only in the crucial metabolic compartments.* (Data from Refs 16–21.)

of BADH activity is in the chloroplast stroma, although there may also be a minor cytosolic isoform. BADH has a preference for NAD^+, and has a similar pH optimum to that of CMO[25]. We have recently cloned cDNAs for BADH from spinach[26], and are currently working on the cDNA cloning of CMO.

The activities of the glycine betaine synthetic pathway enzymes are regulated by osmotic stress. CMO activity is increased threefold by treatment of spinach plants with 200 mM NaCl[24]. Likewise, BADH protein and mRNA levels are increased several-fold by salt treatment[26,27]. At present, it is not known whether control of BADH mRNA amounts is at the transcriptional, or post-transcriptional level, or both.

Choline, the substrate for CMO, is ubiquitous in nature and its biosynthesis is under feedback control in all plants tested[28–30]. A priori, it therefore seems likely that the supply of choline would be adequate to support the additional demand caused by introduction of the glycine betaine biosynthetic pathway. However, this assumption should be treated with caution; species appear to differ in the involvement of soluble versus phospholipid-bound intermediates in choline synthesis, perhaps indicating differences in compartmentation of the pathway or its end product[31,32].

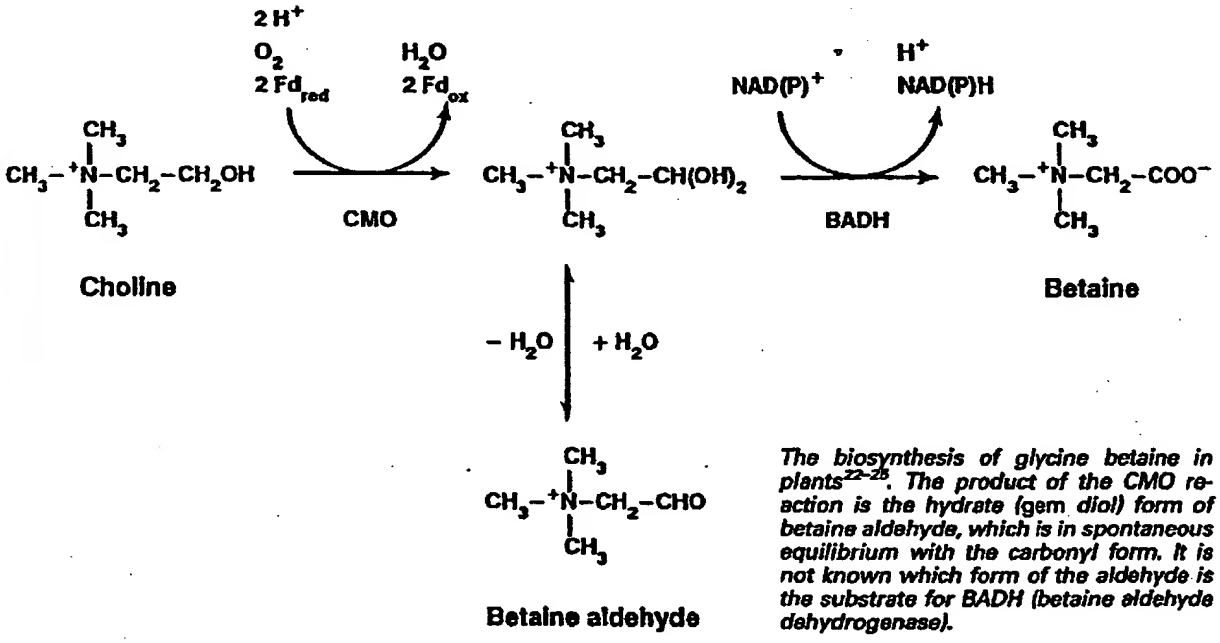
Towards engineering of target species

Glycine betaine accumulation does not occur in many important crop species into which genes can be inserted with current technology. Some of these betaine-deficient species, such as rice, tomato and potato, are probably the most promising targets for genetic engineering; the fact that these have betaine-accumulating relatives makes it more probable that enough choline will be available in the chloroplast to sustain high rates of betaine synthesis, and that the transport mechanisms required to maintain proper compartmentation of betaine will be present.

What might be the effect of introducing the betaine pathway into a non-accumulator on productivity under stress? As yet, this cannot be answered directly. However, comparing biomass yields of wild-type, betaine-accumulating maize genotypes with those carrying a naturally occurring betaine-deficiency mutation showed a yield advantage of about 12% for the betaine-accumulators at a dry site in Mexico (C. Lerma, J. Bolaños, D. Rhodes and A. D. Hanson, unpublished). An effect of this magnitude on production is the most that can reasonably be expected from introducing a single trait[33].

Having selected a target crop species, a number of assumptions must be made in order to proceed (Fig. 5). For example: glycine betaine is osmoprotective in a wide variety of organisms, so it is likely to function similarly in plants that do not naturally accumulate it, though this will need to be proved for each target species. The biosynthesis of glycine betaine from choline is fairly well understood and as genes are isolated for both steps in the pathway, we may assume that they will include the sequences necessary for targeting to the chloroplast. The production of the necessary levels of active enzyme product should be attainable using constitutive promoters already available, although these will probably not reproduce the stress-inducibility which characterizes the natural betaine pathway. Whether correct localization of glycine betaine in the cytosol and chloroplast will occur is not predictable at this point. We do know that non-accumulators such as tobacco show active uptake of supplied glycine betaine[34], although the subcellular

Fig. 4



The biosynthesis of glycine betaine in plants[22–25]. The product of the CMO reaction is the hydrate (gem diol) form of betaine aldehyde, which is in spontaneous equilibrium with the carbonyl form. It is not known which form of the aldehyde is the substrate for BADH (betaine aldehyde dehydrogenase).

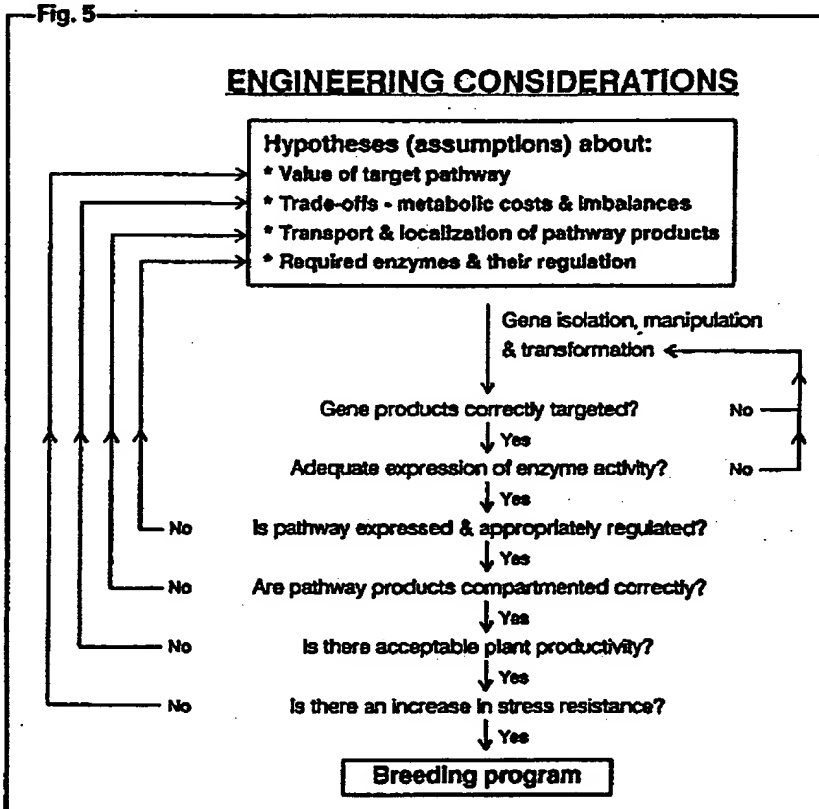
location of the absorbed betaine has not been determined.

Supposing the preceding assumptions hold, then transgenic plants which accumulate betaine in the appropriate compartments will be obtained. Field performance of these transgenic plants will then have to be compared with controls, in the absence and presence of stress. Suppose that performance (yield) under stress is improved, but is poorer in the absence of stress, and that betaine accumulation proves not to be stress-regulated in the engineered plants. Such an outcome would indicate the desirability of a more ambitious engineering project – the engineering of stress-inducibility, which could be achieved by placing the genes of the pathway under the control of an osmotically responsive promoter. Isolation of such a promoter is possible (but has not yet been done), but its proper functioning would probably require *trans*-acting factors as well as signal detection and transducing machinery to be present in the target plant. If the elements of such a network are not present, their identification and isolation would demand much additional basic research.

Possible engineering problems

We have indicated several ways in which engineering glycine betaine accumulation into transgenic plants may not be straightforward, and that

Fig. 5



Engineering considerations in modifying plant stress resistance by biotechnology. It is important to note that for stress resistance, as for many other desirable biochemical traits, the essential metabolites, enzymes and genes involved are not yet completely understood. Therefore, in order to proceed, numerous hypotheses or assumptions must be made. These can only be tested by actually undertaking the engineering of a target species.

the effect on stress resistance is not predictable. Such uncertainties can only be confirmed or dismissed by carrying out some pioneering engineering experiments (Fig. 5). Until the genes for CMO and BADH are introduced and the transgenic plants evaluated, we cannot be certain that these genes are sufficient for betaine synthesis and accumulation. Correct targeting of the enzymes to the chloroplast is another possible obstacle, especially when transferring genes from monocots to dicots[35]. The substrate choline must be present in the chloroplast; this may require *in-situ* synthesis or the proper transport mechanisms, and the known differences in the biosynthesis of choline between plants[31,32] may affect the choline availability. Compartmentation of glycine betaine may depend upon specific transport mechanisms in the chloroplast envelope, tonoplast and plasma membrane, possibly not present in all plants; this can be substantiated only after successful transfer of the biosynthetic pathway.

Successful expression of betaine accumulation might, in itself, create problems. Betaine synthesis could compete detrimentally with choline utilization in phospholipid synthesis, causing an overall poorer performance in the engineered plant. There could also be more general effects resulting from increased carbon and nitrogen partitioning into the betaine synthetic pathway; although calculations suggest that these should be inconsequential[36,37], there is no way of being certain in advance.

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Proline Accumulation as Drought-tolerance Selection Criterion: its Relationship to Membrane Integrity and Chloroplast Ultrastructure in *Nicotiana tabacum* L.

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Summary

The value of proline accumulation as a criterion in selecting for drought-tolerance, was evaluated in four tobacco cultivars of differing drought-tolerance. Proline determination, measurement and calculation of a membrane integrity index (MII), and ultrastructural observations were conducted simultaneously under controlled environmental conditions during the stress period. Water stress of increasing severity (0.2–0.3 MPa d^{-1}) that ranged from light (−0.52 MPa) to severe (−2.51 MPa) was induced by withholding water. A substantial accumulation of proline was observed in all four cultivars, the extent of which correlated positively with their individual drought-tolerance. Ultrastructural observation indicated that the drought-tolerant cultivars mobilized the, more than adequate, store of starch to a greater extent than the drought-sensitive cultivars during stress. Water stress-induced membrane damage occurred earlier and was much more severe in the drought-sensitive cultivars, as their MII-values already rose to between 20.4 % and 23.4 % at a Ψ_L of −1.27 MPa, while those of the drought-tolerant cultivars only reached values of 18.5 % and 20.9 % at a Ψ_L of −1.67 MPa. The Ψ_L at which proline levels start to increase dramatically (the shorter the response time the better) and the end concentrations of proline accumulated, are advocated as criteria to be used in selecting for drought-tolerant tobacco genotypes as early as the F_1- or F_2-generations.

Key words: Nicotiana tabacum L., membrane integrity, proline accumulation, selection criterion, starch content, ultrastructure, water stress.

Abbreviation: MII = membrane integrity index.

Introduction

Within the leaves of many plants subjected to moderate or severe water stress, one striking change in nitrogen metabolism is the accumulation of free proline as a result of net *de novo* synthesis from glutamic acid (Barnett and Naylor, 1966; Boggess et al., 1976). Water stress-induced proline accumulation can account for as much as 1 % of the dry leaf matter in many species; this, however, only takes place if there are adequate carbohydrates present in the tissue (Hsiao, 1973). Proline has been shown to ameliorate the deleterious effects of heat, pH, salt, chemicals and dehydration on enzyme activity and organelle systems (Ahmad et al., 1979; Bowlus and Somero, 1979; Nash et al., 1982; Yancey and Somero, 1979). The mechanism whereby these «compatible» solutes (Schobert and Tschesche, 1978) exert their influence has, however, not yet been elucidated (Stewart, 1989), nor are the molecular mechanisms underlying these effects completely understood. Proline action has been suggested to involve effects on the hydration layer surrounding phospholipids and possibly also its intercalation between phospholipid head groups (Rudolph et al., 1986). Furthermore, it is striking that many of the solutes which accumulate in stressed plants and which have protective

properties are also reported to reduce free radical activity. In this regard it has been indicated that proline can also detoxify free radicals by forming long-lived adducts with them (Floyd and Zs-Nagy, 1984). Just as little is known about the changes which the tonoplast and plasmalemma undergo during desiccation, although leakage studies indicate that some changes do occur (Bewley and Krochko, 1982). Many of these studies have been reviewed by Simon (1974, 1978). What is known, however, is that when enzymes, structural proteins, macromolecular complexes, etc., are desiccated in their native state, the integrity of the molecules and structures can be retained if some water remains associated with them, which prevents the formation of unfavourable conformations (Todd, 1972) or fragmentation (Darbyshire and Steer, 1973).

As stated above, it has been suggested for some time that the often observed accumulation of proline in plant tissues during water stress is an adaptive response (Handa et al., 1986). It can be argued, however, that the mere correlation between accumulation and development of stress is not enough proof that the substance has any adaptive value in postponing stress or increasing stress-tolerance and that the accumulation occurs because of disturbance of normal nitrogen metabolism with the result that any beneficial effects, if such exist, are merely coincidental (Kramer, 1983).

As it is very difficult to prove that these compounds have an important adaptive value, this study was conducted as an attempt to gain some evidence, albeit indirectly, as to whether the ability to accumulate proline in four tobacco cultivars, can be positively correlated with their drought-tolerance, because of its protective membrane stabilizing effects. To achieve the latter, it was deemed necessary to find answers to the following questions: Firstly, does a positive correlation exist between proline accumulation, membrane integrity and the known difference in drought-tolerance of the four tobacco cultivars? Secondly, do the four cultivars contain enough carbohydrates to allow proline accumulation and does the extent to which starch is mobilized during water stress differ among the four cultivars? Thirdly, how extensive are the water stress-induced ultrastructural changes and do they differ in the four cultivars? Finally, which, if any, aspect(s) of proline accumulation may be useful as selection criteria in selecting for drought-tolerance?

Material and Methods

In sequence of increasing drought-tolerance the four cultivars of *Nicotiana tabacum* L. under investigation, were TL33, CDL28, GS46 and ELSOMA. These cultivars were selected due to their differing performances in the field during water stress periods (Personal communication: Dr. C. J. Steenkamp, Tobacco and Cotton Research Institute, Rustenburg, RSA). Seed was allowed to germinate in soil in pots. The young seedlings developed under glasshouse conditions with optimal water application (watered once daily to field capacity). Before the onset of experimentation the plants were moved to a growth room (photon flux density, 400–600 μ mole $m^{-2} s^{-1}$; relative humidity, 35 %) and allowed to acclimatize for a period of 96 hours. During the acclimatization period the plants were watered twice daily to avoid their experiencing any water stress because of the slightly higher growth chamber temperatures. The growth chambers were lit for 13 hours at 25 °C followed by an 11 hour dark period at 16 °C. Experimentation started when the plants were approximately 90 days old and a water stress of increasing intensity (0.2–0.3 MPa d^{-1}) was induced by withholding water. The water stress period lasted for 12 days and ranged from light (−0.52 MPa) to severe (−2.51 MPa). Leaf water potential determinations were done every second day with a Scholander pressure chamber (PMS-instrument, Oregon, USA). Membrane integrity, measured as ion leakage, was carried out as described by Sullivan and Ross (1979) and modified by Blum and Ebercon (1981). Proline concentration was determined by means of a rapid colorimetric method, developed for plant tissue by Bates et al. (1973). All leaf samples were taken at 08:00. The sixth youngest leaf was used in all instances, as this represented a mature, nearly fully expanded leaf, before the onset of senescence.

For ultrastructural investigation samples of 1 mm^3 were cut from the centre of the lamina, lateral to the midrib, while immersed in fixative, and these were immediately transferred to fixative (2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). After evacuation of 15 minutes, fixation was continued for a further 2 hours. The material was postfixed for 1 hour in 0.5 % aqueous osmium tetroxide, dehydrated in acetone and infiltrated with resin (Spurr, 1969). For light microscopy semi-thin sections were made, stained with toluidine blue O (Trump et al., 1961) or toluidine blue O and neofuchsin (Botha et al., 1982). For electron microscopy, thin sections were contrasted with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

Results and Discussion

The calculated MII-valqes (Table 1) of the four cultivars clearly indicata that the membranes of the drought-sensitive cultivars 6TL33 and CDL28) were damaged earlier than those of the drought-tolerant cultivars (GS46 and ELSOMA). This is evident from the fact that the MII-values of TL33 and CDL28 already increased to 20.4 % and 23.4 % respectively at a. Ψ_L of −1.27 MPa whilst the MII-values of GS46 and ELSOMA only rose to similar levels i.e. 20.9 % and 18.5 % respectively at a Ψ_L of −1.67 MPa. Furthermore, at the severe stress level (−2.51 MPa) the water stress-induced membrane damage was found to be approximately 10 % more in the drought-sensitive cultivars. The value of the above-mentioned results is only fully realized when seen in conjunction with the statement of Sullivan and Ross (1979). These authors have been concerned with the relationships between electrolyte leakage following a shock treatment and general ability of plants to tolerate stress for several years. According to them, the evidence they obtained indicated that the degree of membrane stability to stress (as evaluated by ion leakage) correlates well with the tolerance of other plant processes to stress, even if the stress is at levels lower than that needed to measure appreciable ion leakage. These include soluble protein and enzyme resistance to denaturation, maintenance of photosynthesis by intact tissue and other responses to stress (Sullivan and Kinbacher, 1967; Sullivan and Ross, 1979; Kinbacher et al., 1967). In the light of our results (Table 1 and Fig. 1) regarding membrane integrity of the test plants, and because it is the rate of water stress-induced membrane injury that is estimated through the measurement of electrolyte leakage from cells (Blum and Ebercon, 1981), it may be said that not only the membrane

Table 1: Water stress-induced proline accumulation and changes in the membrane integrity index of four cultivars of *Nicotiana tabacum* L., which differ with respect to their drought-tolerance.

Cultivar	Ψ_L, MPa	Proline conc., mmol kg^{-1} dry mass	% of control	MII % of control
TL33	−0.52	0.60±0.01	100.0	0.0±0.0
	−0.77	1.91±0.04	318.5	3.8±0.6
	−1.27	3.40±0.9	566.7	20.4±0.7
	−1.67	3.72±0.4	620.0	22.4±0.5
	−1.97	9.50±0.3	1583.3	30.4±0.4
	−2.32	18.55±0.3	3019.7	38.5±0.8
	−2.51	35.66±0.7	5943.3	51.1±0.9
CDL28	−0.52	0.81±0.02	100.0	0.0±0.0
	−0.77	1.99±0.01	245.7	4.5±0.3
	−1.27	3.30±0.3	407.4	23.4±0.8
	−1.67	5.45±0.4	672.8	28.4±0.9
	−1.97	5.82±0.9	718.5	32.3±0.9
	−2.32	16.26±0.4	2007.4	36.1±0.5
	−2.51	32.22±0.3	3977.7	52.8±0.6
GS46	−0.52	0.17±0.01	100.0	0.0±0.0
	−0.77	1.19±0.04	700.0	6.2±0.1
	−1.27	2.12±0.03	1247.1	7.4±0.8
	−1.67	6.83±0.5	4017.6	18.5±0.7
	−1.97	14.16±0.2	8329.4	29.4±0.2
	−2.32	30.20±0.8	7764.7	40.6±0.7
	−2.51	41.90±0.2	4647.1	42.9±0.4
ELSOMA	−0.52	0.38±0.01	100.0	0.0±0.0
	−0.77	1.59±0.02	418.0	6.8±0.9
	−1.27	2.05±0.9	539.5	8.9±0.5
	−1.67	11.71±0.3	3081.6	20.9±0.3
	−1.97	23.72±0.2	6242.1	26.7±0.6
	−2.32	44.78±0.5	1784.2	32.5±0.4
	−2.51	47.62±0.7	2531.6	41.1±0.5

MII = $1-[1-(T_1/T_2)/1-(C_1/C_2)]$ X 100 where according to Blum and Ebercon (1981), T and C refer to mean of treatment and controls, respectively, and subscripts 1 and 2 refer to initial and final conductivities, respectively.

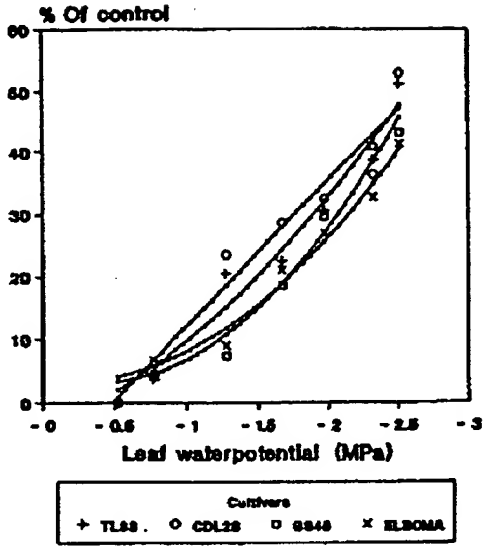

Fig. 1: Effect of water stress on the membrane integrity of four cultivars of *Nicotiana tabacum* L. of differing drought-tolerance.

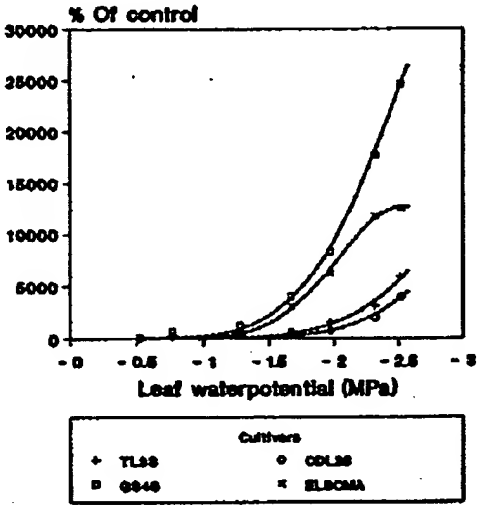

Fig. 2: Water stress-induced proline accumulation in four cultivars of *Nicotiana tabacum* L. which differ with respect to their drought-tolerance.

systems of the drought-sensitive cultivars, but also their physiological processes in general were damaged earlier. Furthermore, the extent of the water stress-induced damage, as reflected by the steeper slope of the MII-curves (Fig. 1), was much more severe in these cultivars. In keeping with the idea that the water stress-induced membrane damage was much more severe in the drought-sensitive cultivars, it should also be noted that at a Ψ_L of −2.51 MPa, membrane damage in these cultivars was irreversible. Dhe latter statement is supported by the fact that the MII-values represent the percentage of membrane injury due to desiccation (Blum and Ebercon, 1981) and these values of the drought-sensitive cultivars exceeded 50 % at a Ψ_L of −2.51 MPa (Table 1).

According to Bewley and Krochko (1982) direct evidence that species specific differences in membrane composition or protein structure contribute to the tolerance of plants is generally lacking. This view is shared by Schwab and Heber (1984) who concluded that drought-tolerance cannot be explained by a particular membrane structure which makes the membrane insensitive to water stress, but must rather, though it may only be in part, be attributed to the composition of the membrane surroundings, with special reference to membrane compatible solutes. In this regard it is interesting to note that Rudolph et al. (1986) found that in the presence of trichalose, sucrose, proline and glycine betaine, ion leakage is prevented.

In accordance with the results obtained by several authors (Bogges and Stewart, 1980; Handa et al., 1986) in several species, our results showed a dramatic increase in the free proline levels of all four the tobacco cultivars (Fig. 2). At a Ψ_L of −0.77 MPa the proline levels of all four the tobacco cultivars already deviated significantly from the control values. The proline concentrations of the drought-sensitive

cultivars were 1.91 and 1.99 µmoles gram^{-1} respectively, which is more than double the proline concentration in the unstressed tissue, while the proline concentration in the drought-tolerant cultivars had already risen to 1.19 and 1.59 µmoles gram^{-1}, which is four times more dhan the proline concentration in the unstressed tissue. The base proline levels of the drought-tolerant cultivars proved to be lower than those of the drought-sensitive cultivars (Table 1). Of greater importance, however, is the fact that the drought-tolerant cultivars were able to accumulate roline earlier and to much higher end concentrations. At a Ψ_L of −1.67 MPa the proline concentrations of both drought-sensitive cultivars were just a little more than six times those of the controls, while that of GS46 was 40 times higher and that of ELSOMA, 30 times higher than their respective controls. At the severe stress level of −2.51 MPa the amount of proline accumulated by ELSOMA was more than three times higher than that of both drought-sensitive cultivars and that of

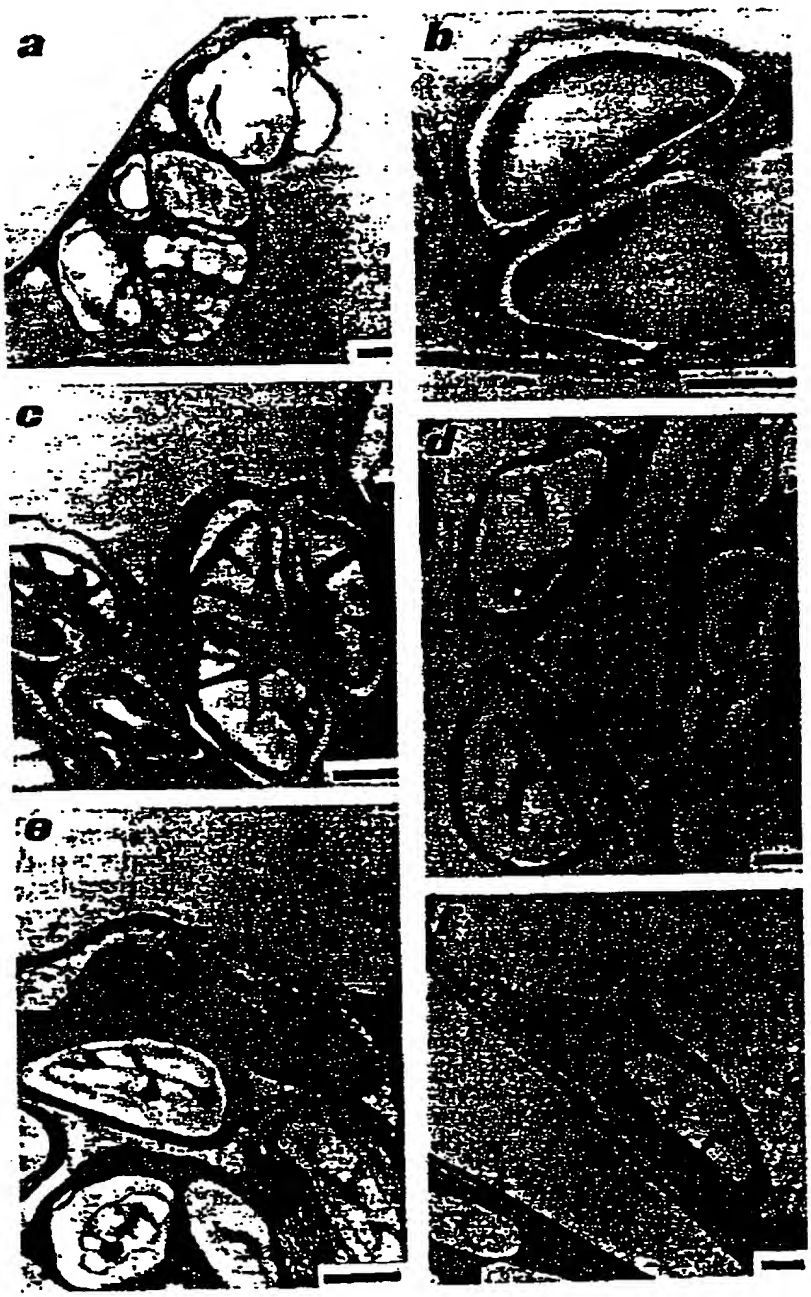

Fig. 3: Differential water stress-induced ultrastructural changes in four tobacco cultivars of differing drought tolerance: (a) CDL28 (Ψ_L = −0.52 MPa); (b) ELSOMA (Ψ_L = −0.52 MPa); (c) CDL28 (Ψ_L = −1.97 MPa); (d) ELSOMA (Ψ_L = −1.97 MPa); (e) TL33 (Ψ_L = −2.51 MPa); (f)ELSOMA (Ψ_L = −2.51 MPa). Bar = 1 µm.

GS46 more than four times higher than both the drought-sensitive cultivars (Table 1). With reference to the latter two sets of results, it should be noted that Hsiao (1973) stated that sketchy indications are that the level of proline may be insensitive to mild stress and that accumulation only takes place if there are adequate carbohydrates in the tissue. The accumulated proline apparently came from *de novo* synthesis (Thompson et al., 1966) with glutamate as precursor (Morris et al., 1969) and there is evidence to indicate that carbohydrates are the eltimate source of the skeleton (Stewart et al., 1966).

With regard to carbohydrate reserves of the test plants the following observations relating to starch grains in the chloroplasts were made. Special attention was, however, paid to the state of the chloroplast envelope membranes as well as to that of the inner thylakoids in this investigation, as according to Ferrari-Iliou et al. (1984) chloroplasts can be regarded as the most fragile organelles in the cell. In the unstressed leaves of all four cultivars the presence of large starch grains was striking, although the leaves were sampled after only 2–3 hours of exposure to light (Figs. 3 a and 3 b). The grana were found to be clearly defined, with parallel thylakoids and little dilatation of the intergranal spaces. Osmiophilic globules, some appearing more osmiophilic than others, occurred in most of the chloroplasts studied.

After water had been withheld for 8 days (Ψ_L = −1.97 MPa), the number and size of the starch grains of the chloroplasts of leaves of cultivars TL33 and CDL28 (Fig. 3 c), showed little change from the original situation observed in the unstressed leaves. The chloroplast envelope membranes in some cells of cultivar TL33 were not clearly defined, while in others they were still intact. Large lipidic globules were present in some of the chloroplasts. Granal and stromal thylakoids were severly disrupted in some chloroplasts. The chloroplast membranes in leaves of CDL28 were still intact and the position of the plasmalemma with regard to the cell wall showed little change, although signs of shrinking of cytoplasm were discernible in some cells. Few vesicular structures were present in the cells. Stromal thylakoids showed some signs of dilatation in some of the cells, but the grana were still intact. The size of starch grains decreased in many chloroplasts in the leaves of cultivars ELSOMA and GS46 (Fig. 3 d). This was especially apparent in the case of GS46, where many of the chloroplasts lost their original bulging appearance due to large starch grains observed in all unstressed leaves. In leaves of cultivar ELSOMA, vesicular structures were present in some of the vacuoles and signs of plasmolysis could be observed. The granal and stromal thylakoids were still intact as were the chloroplast membranes (Fig. 3 d). Cells with granular vacuolar content were observed. Chloroplast membranes in leaves of GS46 were still intact and the thylakoids appeared normal in most of the cells investigated. Vesicular structures were observed in many of the cells. One of the most noticeable features of these cells was the granular or fibrillar material which collected in some of the vacuoles (Fig. 4). This was also discernible in the sections prepared for light microscopy and was not observed in any of the other cultivars at this stage.

After water had been withheld for 12 days (Ψ_L = −2.51 MPa), large lipidic globules, some more osmiophilic than others, appeared in chloroplasts of all the cultivars investigated (Fig. 3 e). Leaves of cultivar TL33 still contained starch grains in most of the chloroplasts (Fig. 3 e). The starch grains were smaller than those present in unstressed leaves. Cytosolic damage was very severe in some cells where vesicular material collected in large parts of the cell. Large osmiophilic accretions appeared in the degenerated cells. In severely damaged chloroplasts signs of bulging of the outer chloroplast membrane were observed. Some of the chloroplasts assumed a curved shape (Fig. 3 e) and the cells showed signs of plasmolysis. Leaves of cultivar CDL28 still contained cells with intact thylakoids. Some of the chloroplasts contained starch grains smaller than those observed in unstressed leaves, while other chloroplasts were almost or completely depleted of starch. This could also be observed by light microscopy. Chloroplast membranes were still intact. Fibrillar material collected in the vacuoles of some of the cells, where the tonoplasts appeared to be poorly defined or damaged. Vesicular structures were present in the vacuoles of some of the cells. Few signs of severe plasmolysis were detected. Leaves of cultivar ELSOMA contained chloroplasts with smaller starch grains (Fig. 3 f). Fibrillar or granular material collected in the vacuoles while few vesicular structures were observed in the vacuoles. Chloroplast envelope membranes were still intact, although the first signs of granal and especially stromal thylakoid dilation were apparent in some of the cells. Signs of plasmolysis and curved chloroplasts were observed in some of the cells (Fig. 3 f). In leaves of cultivar GS46, the stromal and granal thylakoids appeared somewhat dilated but all membranes investigated were still intact. Small starch grains were present in the chloroplasts. Fibrillar or granular material was present in the vacuoles (Fig. 4). These cells showed little sign of plasmolysis.

Fig. 4: Distinctive water stress-induced appearance of granular or fibrillar material in the vacuole of cultivar GS46 after a Ψ_L of −1.97 MPa was reached. Bar = 1 µm.

Thus, although no complete loss of starch was observed at any stage during water stress, in contrast to the situation reported by Giles et al. (1976) in water stressed leaf tissue of *Sorghum bicolor*, a decline in the starch content was observed

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Pyrroline-5-Carboxylate Reductase Is in Pea (*Pisum sativum* L.) Leaf Chloroplasts[1]

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ABSTRACT

Proline accumulation is a well-known response to water deficits in leaves. The primary cause of accumulation is proline synthesis. Δ¹-Pyrroline-5-carboxylate reductase (PCR) catalyzes the final reaction of proline synthesis. To determine the subcellular location of PCR, protoplasts were made from leaves of *Pisum sativum* L., lysed, and fractionated by differential and Percoll density gradient centrifugation. PCR activity comigrated on the gradient with the activity of the chloroplast stromal marker NADPH-dependent triose phosphate dehydrogenase. We conclude that PCR is located in chloroplasts, and therefore that chloroplasts can synthesize proline. PCR activities from chloroplasts and etiolated shoots were compared. PCR activity from both extracts is stimulated at least twofold by 100 millimolar KCl or 10 millimolar $MgCl_2$. The pH profiles of PCR activity from both extracts reveal two separate optima at pH 6.5 and 7.5. Native isoelectric focusing gels of samples from etiolated tissue reveal a single band of PCR activity with a pI of 7.8.

Induction of proline accumulation by water deficit is a well-known, but little understood, phenomenon in plant stress physiology. Proline accumulation is caused primarily by increased synthesis from glutamic acid; the biosynthetic pathway is postulated to be analogous to that which converts glutamic acid to proline in *Escherichia coli* (21). The first two enzymes of this pathway have yet to be defined in plant extracts. The third and final enzyme PCR,[2] has been measured from several plant sources (12, 13), and Krueger *et al.* (10) have purified it to apparent homogeneity from wilted barley leaves.

The subcellular location of proline biosynthesis has not been clearly established. The involvement of light in this process has been indicated. Noguchi *et al.* (17) have shown that inhibition of PSII inhibits proline synthesis in tobacco leaf discs. Rajaopal *et al.* (20) have shown that the pattern of proline accumulation in drought-stressed wheat parallels the pattern of diurnal change in light intensity. PCR activity has been reported in chloroplast-enriched fractions from tobacco leaves (16), but Kohl *et al.* (9) found that in soybean root nodules this enzyme is in the cytosol and not in plastids. To obtain an indication of the subcellular location of proline biosynthesis in leaves, we have investigated the subcellular location of PCR, the only proline biosynthetic enzyme for which a reliable assay in higher plants is available. Our results indicate that, like many other amino acid biosynthetic enzymes, PCR is located in the chloroplasts. We have also compared the properties of PCR from leaves and from etiolated shoots and find that they are similar.

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[2] Abbreviations: PCR, Δ¹-pyrroline-5-carboxylate reductase; MMT, Mes, Mops, Tricine; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHAPSO, 3-([3-cholamidopropyl]-diethylammonio)-2-hydroxy-1-propanesulfonate; GK, γ-glutamyl kinase; GPR, γ-glutamyl phosphate reductase; TPDH, triose phosphate dehydrogenase.

MATERIALS AND METHODS

Plant Material

Peas (*Pisum sativum* L. var Argenteum) were grown in soil flats in a growth chamber under the following conditions: 16 h light, 8 h dark, 20°C, 270 μmol s^{-1} m^{-2} combined fluorescent and incandescent light. Plants were watered with Hoagland solution (7) every third day and were allowed to wilt for 1 d each week. The wilting ensured that increased proline synthesis was stimulated. The Argenteum variety was used because its leaf epidermis is easily peeled away. Protoplasts were prepared from peeled leaves. Etiolated peas (*P.* sativum L. var Progress No. 9) were grown in coarse vermiculite at 30°C in darkness for 9 to 12 d and watered with deionized water. Progress No. 9 was used for experiments with etiolated tissue because sufficient Argenteum seed was not available.

Protoplast Preparation

Pea protoplasts were prepared from 4-week-old leaves of the Argenteum variety (24). Plants were destarched by placing them in darkness for 24 h. Adaxial epidermises were peeled and 40 leaves were floated on protoplast buffer (500 mM sorbitol, 5 mM Mes-KOH, 1 mM $CaCl_2$, [pH 6.0]) containing wall digesting enzymes (2% [w/v] Onozuka cellulase, 0.5% [2/v] Macerozyme, 1% [w/v] hemicellulase) and 0.2% (w/v) BSA in 8.5 cm Petri dishes in darkness at 30°C. After 1 h, the digestion medium was aspirated and 10 mL of protoplast buffer was added to each dish. Protoplasts were released by gentle rocking and decanted into a beaker, then another 10 mL of protoplast buffer was added, and the remaining protoplasts were decanted. Protoplasts were collected by centrifugation in a swinging bucket rotor at $100g_{max}$ for 1 min at 4°C. The supernatant was aspirated and discarded. Protoplasts were resuspended in 5 mL of chloroplast buffer (300 mM sorbital, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.2% [w/v] BSA, 50 mM Mops-KOH [pH 7.2]).

Protoplast Fractionation

Protoplasts were ruptured by passing the suspension three times through 20-μm nylon mesh at the end of a syringe barrel. Microscopic examination revealed complete lysis of all protoplasts after this treatment. The resulting suspension was centrifuged in a Sorvall HB-4 swinging bucket rotor at 250g_{max} for 2 min. Pellets, which contained both damaged and intact chloroplasts, were resuspended in 3 mL chloroplast buffer and overlaid on a Percoll (Pharmacia) gradient. The gradient was generated by mixing 15 mL Percoll with 15 mL 2× chloroplast buffer in 50-mL polypropylene tubes and centrifuging at 40,000g_{max} in a Sorvall SS-34 fixed angle rotor for 30 min; the rotor was stopped without the brake. The overlaid gradient was centrifuged at 8,000g_{max} for 20 min in a Sorvall HB-4 swing-out rotor without the brake. A 12-cm-long, 20-gauge needle was inserted into the gradient to the bottom of the tube, and the gradient was fractionated into 1.6-mL aliquots using a peristaltic pump.

Extraction of PCR

Because Percoll interfered with enzyme assays, protein was precipitated from each Percoll gradient fraction by addition of 2.4 mL of a 50% (w/v) polyethylene glycol (3,500 average molecular mass) solution buffered with 50 mM Mops-KOH (pH 7.2). Samples were vortexed, incubated 10 min, and centrifuged in a Sorvall SM-24 fixed angle rotor at 20,000g_{max} for 10 min. Supernatants were aspirated and the pellets resuspended in 0.2 mL of 20 mM Tricine-KOH (pH 8.0), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 20% (v/v) glycerol.

Etiolated pea shoots were harvested and stored at −20°C. Five hundred g of shoots were homogenized in 500 mL grinding buffer (100 mM Mops-KOH, 1 mM EDTA, 20 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5% [w/v] insoluble PVP) with a Polytron tissue homogenized (Brinkmann Instruments) at 4°C for 5 min. The slurry was filtered through four layers of cheesecloth. After filtration, the extract was brought to 30% saturation with $(NH_4)_2SO_4$ at 40°C, incubated 15 min, and centrifuged in a Sorvall GSA rotor at 25,000g_{max} for 10 min. The supernatants were pooled, brought to 60% saturation with $(NH_4)_2SO_4$, incubated 15 min, and centrifuged again. The pellets were resuspended in 5 ml 20 mM Tricine-KOH (pH 8.0) and desalted on a Sephadex G-25 column (2.5 × 28 cm) equilibrated with the same buffer. This preparation was made 20% (v/v) with glycerol and stored at −20°C.

For assays of crude chloroplast preparations, PCR was rapidly extracted from chloroplasts by the following modification of the procedure used for gradient purification of chloroplasts. The 250g pellet from a lysed protoplast suspension was resuspended in 6 mL of chloroplast buffer and recentrifuged at 250g for 2 min in a Sorvall HB-4 rotor. This pellet was resuspended in 2 mL of lysis buffer (20 mM Tricine [pH 8.0], 0.1 mM PMSF, 1 mM DTT) plus 1% (w/v) CHAPSO. After 10 min at 4°C, the sample was centrifuged in a Sorvall SS-34 rotor at 43,500g_{max} for 15 min at 4°C. The supernatant, which was yellow-green, was assayed.

Assays

The following assays were performed as previously described. NADP$^+$-dependent triose phosphate dehydrogenase was used as a stromal marker (11), Chl as a thylakoid marker (11), Cyt c oxidase as a mitrochondrial marker (1), and catalase as a peroxisome marker (2). The general PCR assay buffer contained 50 mMTricine-KOH (pH 8.0), 1 mM DTT, 200 μM NADH, and 2 mM D,L-Δ^1-pyrroline-5-carboxylate (D,L-P5C). For the subcellular fractionation experiments the buffer also contained 100 mM KCl and 0.01% (w/v) Triton X-114. D,L-P5C was prepared by the method of Williams and Frank (26). P5C-dependent NADH oxidation was measured at 340 nm. The extinction coefficient of NADH (6.2 mM^{-1} cm^{-1}) was used to calculate PCR activity. Kinetic parameters were determined by iterative fitting of the Michaelis-Menten equation (25).

Isoelectric Focusing

A modification of a previously described method (15) was used. Native isoelectric focusing was carried out in gels that were poured and run in a Mighty Small electrophoresis apparatus (Hoefer). Gels contained 4% (w/v) acrylamide, 0.0016% (w/v) methylene-*bis*-acrylamide, 1% (v/v) NP-40, 10% (v/v) glycerol, and 5% (v/v) Pharmalytes (pH 3–10). Gels were run at 200 V for 2 h followed by 400 V for 2 h. To locate PCR, gels were rocked for 2 h in 50 mL of 40 mM CAPS-HCl (pH 8.5), 100 mM L-proline, 1 mM NAD$^+$, 1 mM $Mg(OAc)_2$, 100 mM KOAc, 300 μg/mL nitroblue tetrazolium, 20 μg/mL phenazine methosulfate. This staining system gives purple bands by the reverse (proline dehydrogenase) reaction of PCR, which is active at pH >9. Two-mm-thick gel slices were also assayed spectrophotometrically for PCR activity in the forward reaction. Slices were incubated in PCR assay buffer for 15 h. Reverse activity of the same samples were measured spectrophotometrically as previously described (12). In these assays forward PCR activity was fivefold greater than reverse activity.

RESULTS

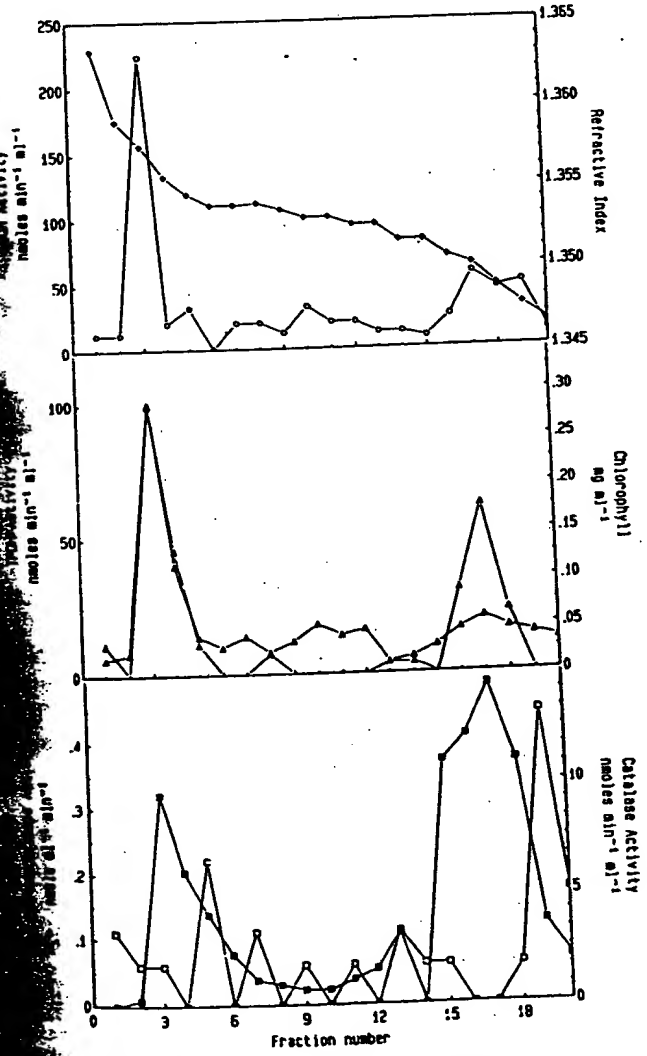
Subcellular Localization of PCR Activity

A crude chloroplast suspension prepared from lysed protoplasts contained 25% of the PCR activity in the protoplast suspension (Table I). The suspension contained comparable proportions of two chloroplast markers, NADP$^+$-TPDH activity (15%) and Chl (19%). This result suggests that PCR may be localized in plastids; however, the suspension also contained significant amounts of activity of a mitochondrial marker enzyme, Cyt c oxidase (6% of total activity), and a peroxisomal marker enzyme, catalase (13% of total activity). When this chloroplast-enriched preparation was fractionated by isopycnic Percoll density gradient centrifugation, the maximum PCR activity coincided with the maximum TPDH activity and Chl concentration (fraction 3, Fig. 1). There was almost no Cyt c oxidase activity in this region of the gradient, but some catalase activity was apparent. The proportion of catalase activity recovered in fraction 3 was only one-third the proportion of chloroplast markers and PCR recovered in

Table I. *Distribution of PCR and Markers in Subcellular Fractions from Pea Protoplasts*

Marker	Total Units[a]			Percent of Total Along Gradient[b]	
	3 mL protoplast suspension	3 mL chloroplast suspension	Total along Percoll gradient[c]	Fraction No. 3	Fraction No. 17
PCR	0.8	0.20	1.02	35	9
TPDH	3.3	0.50	0.54	29	6
Chl	5.0	0.95	1.44	33	21
Cyt c oxidase	6.3	0.35	0.003	3	0
Catalase	4.2	0.55	0.14	11	17

[a] 1 unit = 1 μmol min^{-1} for enzymes and 1 mg for Chl. [b] Numbers in the third column represent 100%. [c] Sum of activities in all fractions.



1. Distribution of PCR activity and subcellular markers along gradient: PCR (○), refractive index (♦), triose phosphate genase (△), Chl (▲), Cyt oxidase (□), and catalase (■). The samples contained 60 mg protein. Fraction 1 is the bottom adient.

that fraction (Table I). The lower recoveries of Cyt oxidase and catalase indicate that fraction 3 contained lesser proportions of mitochondria and peroxisomes than of chloroplasts. Moreover, the distribution of PCR activity in the gradient as a whole coincided more closely with the distribution of TPDH than with that of catalase or Cyt oxidase.

In three of seven replicate gradients (data not shown), PCR activity was prominent in the ruptured chloroplast region (fraction 17). This distribution suggests that PCR is associated with thylakoid membranes. Several nonionic detergents were added to the chloroplast lysis buffer to test their ability to dissociate interactions between PCR and thylakoids. The yield of PCR activity in the soluble faction from chloroplasts was increased twofold by NP-40, CHAPS, or CHAPSO (data not shown).

Whole etiolated shoots yielded 100-fold greater PCR activity than chloroplasts from green leaves. When etioplasts were purified from pea shoots on a Percoll gradient, PCR activity (42 nmol min^{-1} mg $protein^{-1}$) was detected, but the yield was less than 1% of the PCR activity of whole shoot extracts. The yield of etioplasts was itself very low: only 1.3% of the total $NADP^+$-TPDH activity of etiolated shoots was present in the etioplast fraction (102 nmol min^{-1} mg $protein^{-1}$). These results suggest that PCR is present in etioplasts, but do not provide conclusive evidence as to its subcellular distribution in etiolated shoots.

Kinetic Properties of PCR

The specificity of chloroplast PCR for pyridine nucleotide cofactors was investigated. At pH 7.5 and in the presence of 2 mM D,L-P4C, the enzyme had apparent K_ms for NADPH and NADH of 0.12 mM and 0.19, respectively, whereas V_{max} was greater with NADPH (0.19 μmol min^{-1} mg $protein^{-1}$) than with NADH (0.15 μmol min^{-1} mg $protein^{-1}$). The kinetic parameters of PCR from etiolated shoots were also examined. PCR from etiolated shoots had apparent K_ms for NADPH and NADH of 0.1 mM and 0.43 mM, respectively. The V_{max} was greater with NADH (3.2 μmol min^{-1} mg $protein^{-1}$) than with NADPH (0.83 μmol min^{-1}).

There were two pH optima for PCR activity in chloroplast preparations, at pH 6.5 and at pH 7.5 (Fig. 2A). These optima are also present in PCR prepared from etiolated pea shoots (Fig. 2B) and similar optima were observed with both enzymes when NADPH was the substrate (data not shown).

Salt stress causes proline to accumulate (3, 8). Light increases the Mg^{2+} concentration in the stroma *in vivo* (19). The effects of salts on PCR activity were investigated to test the hypothesis that proline biosynthesis can respond to changes in ion concentration. One hundred mM KCl (Fig. 3) or 10 mM $MgCl_2$ (Fig. 4) caused an approximately twofold increase in the PCR activity of both chloroplast and etiolated preparations. Potassium acetate, NH_4Cl, and NH_4OAc stimulated PCR activity to the same extent as KCL (Table II). Maximal stimulation was observed at concentrations between 50 and 100 mM KCl. Activity declined at concentrations above 100 mM; at 200 mM KCl, activities were only slightly higher than the control activity of the chloroplast enzyme increased to a maximum at 10 mM $MgCl_2$ and did not differ between 10 and 20 mM (Fig. 4). Activity of the enzymes from

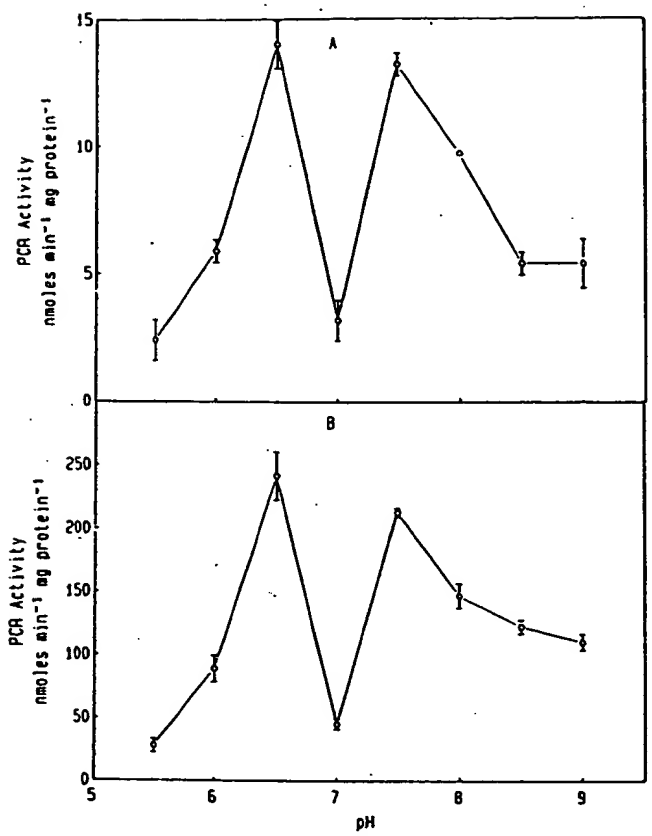


Figure 2. Effect of pH on PCR activity from pea chloroplasts (A) and etiolated pea shoots (B). MMT buffer used for the pH curve contained 50 mM Mes, 50 mM Mops, and 50 mM Tricine. HCl or KOH was used to adjust pH. Except for the change in buffer, the general PCR assay conditions were used, NADH being the substrate. Results are the means and standard errors of four assays.

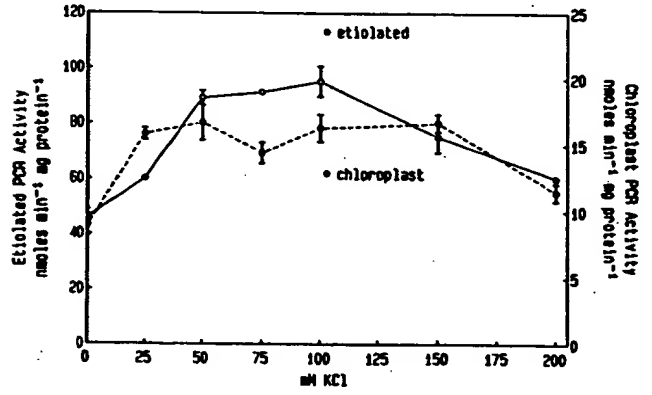


Figure 3. Effect of KCl on PCR activity from pea chloroplasts (●) and etiolated pea shoots (○). Assay was as described in "Materials and Methods," but KCl was added. Results are the means and standard errors of four assays.

etiolated tissue increased with increasing $MgCl_2$ concentration to a maximum at 5 mM, but activity at 10 and 20 mM was lower than it was at 5 mM (Fig. 4). Sucrose and sorbitol of osmolalities equal to that of 100 mM KCl did not cause any significant stimulation (Table II). Potassium acetate, ammonium chloride, and ammonium acetate stimulated PCR activity to the same extent as KCl (Table II). Manganese chloride, calcium chloride, and magnesium acetate caused the same stimulation as magnesium chloride (Table II). The effects of KCl and $MgCl_2$ were not additive at concentrations that gave maximal stimulation alone (Table II).

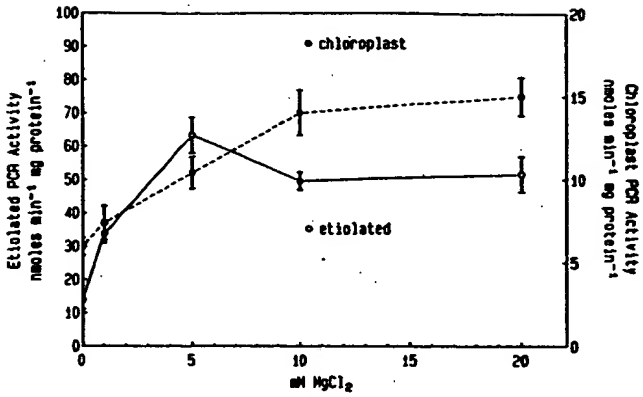


Figure 4. Effect of $MgCl_2$ on PCR activity from pea chloroplast (●) and etiolated pea shoots (○). Assay was described in "Materials and Methods," but $MgCl_2$ was added. Results are the means and standard errors of four assays.

Table II. *In Vitro Effects of Salts and Sugars on PCR Activities[a] from Pea Leaf Chloroplasts and Etiolated Pea Shoots*

Treatment	Chloroplast	Etiolated Shoot
	nmol min⁻¹ mg protein⁻¹[b]	
Control[b]	4[c] (1.0)	35 (7)
Monovalent Cations		
+100 mM KCl	9 (0.2)	138 (31)
+100 mM KOAc	9 (1.1)	127 (3)
+100 mM NH_4Cl	10 (0.6)	162 (3)
+100 mM NH_4OAc	8 (1.2)	142 (39)
+200 mM Sucrose	3 (0.5)	39 (5)
+200 mM Sorbitol	3 (0.6)	39 (4)
Divalent cations		
+10 mM $MgCl_2$	13 (0.7)	132 (19)
+10 mM $MnCl_2$	14 (1.3)	170 (10)
+10 mM $CaCl_2$	13 (1.3)	164 (23)
+10 mM $Mg(OAc)_2$	14 (0.7)	117 (33)
Combined cations		
+10 mM $MgCl_2$ + 100 mM KCl	8 (1.0)	147 (8)

[a] Mean (SE). Each value represents the mean of four assays. [b] Assay described in "Materials and Methods."

Isoelectric Focusing

Separation of the pH 6.5 and pH 7.5 PCR activities from etiolated shoots was attempted by native isoelectric focusing. One zone of activity ($R_f = 0.44$, pI 7.8) contained PCR activity (Fig. 5). Activity of the reverse PCR reaction (P5C dehydrogenase activity, pH 9.5) produced bands of purple precipitate in the same region. The results were the same when $NADP^+$ was the substrate.

DISCUSSION

Noguchi *et al.* (16) described the localization of PCR activity in a chloroplast-enriched fraction from tobacco leaves.

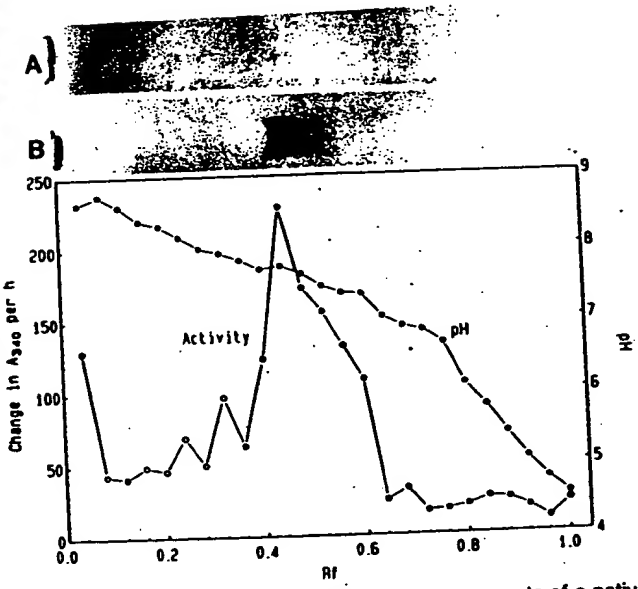


Figure 5. PCR activity (○) and pH (●) in 2 mm segments of a native isoelectric focusing gel. R_f = position of proteins in segment relative to position of a pI marker, methyl red. The general PCR assay was used with the addition of 100 mM KCl. This gel was stained for proline dehydrogenase activity (pH 9.5), (A) without proline, and (B) with proline.

...hl *et al.* (9), however, found that in soybean nodules, PCR ...as localized in the cytosol and not in plastids. The results in Figure 1 and Table I demonstrate that in green pea leaves PCR is present in chloroplasts, since the distribution of PCR activity when leaf protoplasts are fractionated most closely resembles the distribution of the stomal marker $NADP^+$-triose phosphate dehydrogenase. Most significantly, PCR activity was most abundant in the same fraction of Percoll gradients as TPDH. About 25% of the PCR activity measured in protoplast suspensions (Table I, column 1) was recovered in chloroplast suspensions (Table I, column 2), while the recovery of intact chloroplasts from protoplasts was approximately 15 to 20% based upon the recovery of $NADP^+$-TPDH and ...l. Because the chloroplast-to-protoplast ratio for PCR activity is greater than the same ratios for chloroplast markers, ...ppears that PCR in plastids can account for all of the pea ... PCR activity. This distribution contrasts with the localization of PCR activity in the cytosol of soybean root nodules ... PCR from pea leaf chloroplasts uses both NADH and ...ADPH as electron donors, but has a lower K_m and higher ... for NADPH. Thus, like PCR from barley (10) and ...acco leaves (16), it is more active with NADPH. This ...ecifity is consistent with the chloroplast localization of this ...zyme, although the enzyme from soybean root nodules is ... more active with NADPH except at very high cofactor ...ncentrations (9).

...reliminary evidence indicates that PCR is present in etio... ...sts from etiolated pea shoots. Although PCR from etiolated ...oots has a lower K_m for NADPH than for NADH, its V_{max}/... ...tios with the two cofactors indicates that it is more active ... NADH than with NADPH. Thus, the most abundant ... of the enzyme in etiolated shoots may be different from ... leaves.

The bimodal pH curve for pea PCR is analogous to that reported previously for PCR in extracts from etiolated barley (*Hordeum vulgare*) and etiolated mung bean (*Vigna radiata*), both of which give broad pH-activity curves with optima or shoulders at pH 6.4 and 8.0 (4). The bimodal pH curve was also observed when NADPH was used as the cofactor. The pH 7 minimum is not an artifact, because the pH profile was reproducible with batches of MMT buffer prepared three different times. The bimodal curve could be produced by a single enzyme or two isoforms. If there are two forms in green leaves, both are probably present in chloroplasts. The ratio of the pH 6.5 to pH 7.5 activities in chloroplast-enriched preparations was equal to the ratio in whole etiolated shoot extracts. PCR in whole protoplast extracts from green leaves produced a broad pH curve. Activities at pH 6.5 and 7.5 were similar (data not shown), as in the chloroplast-enriched fraction and in etiolated shoots. There was no minimum apparent at pH 7.0, but the enzyme was difficult to assay precisely in whole protoplast extracts because of its low activity. This investigation focused on the PCR activity with the pH 7.5 optimum.

The native isoelectric focusing gel revealed only one region of PCR activity, with a pI of 7.8; the same pattern of PCR activity was detected with NADH and NADPH. Thus, it appears that if there are to isoenzymes in etiolated shoots, they have the same pI. An alternative explanation is that one of the isoenzymes is inactivated during native isoelectric focusing. This activity does not represent the activity of mtiochondrial proline dehydrogenase: the mitochondrial proline dehydrogenase is not active at pH 9.5 (4), and it does not donate electrons to NAD^+. The genetic basis for the pH dependence of PCR activity remains uncertain.

The stimulation of PCR by salts is unclear but it appears to be a function of ionic charge. Because potassium ion concentrations in chloroplasts are normally around 100 mM, the enzyme should be fully active *in vivo*. Changes in stromal pH between light and dark occur in the range that includes the pH optima observed in this work. Stromal pH in the dark is approximately 7, corresponding to the trough in activity; stromal pH approaches 8 in the light, so that the pH 7.5 activity would function in the light. Proline synthesis occurs in leaves in both light and darkness (6, 16, 17, 23), but is stimulated in light.

A role for proline as a redox shuttle molecule has been established for some mammalian tissues (20) and has been proposed for nitrogen fixing nodules (9). The existence of such a shuttle mechanism in leaves needs to be investigated. If such a shuttle operates in leaves under nonstressed conditions, then the localization of PCR in chloroplasts would function to transport reducing potential to mitochondria in the form of proline. To test such a redox shuttle hypothesis, a mechanism that transports proline across the chloroplast envelope needs to be identified. An uncoupled redox shuttle mechanism would contribute to proline accumulation. If water deficit caused such a shuttle to become uncoupled by decreasing proline oxidation without decreasing proline synthesis, then proline accumulation could be a symptom of metabolic dysfunction. Because proline has biocompatible characteristics and proline accumulation is not toxic, metab-

olism may be temporarily shunted in this direction until homeostasis is regained.

Since the product of P5C reductase is proline, the results of this investigation provide evidence that, like many other amino acids, proline is synthesized in chloroplasts. It will be of considerable interest to determine whether the enzymes catalyzing conversion of glutamate to P5C have the same subcellular location.

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Osmoregulation of a Pyrroline-5-Carboxylate Reductase Gene in *Arabidopsis thaliana*[1]

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In *Arabidopsis thaliana* (L.) Heynh. proline can account for up to 20% of the free amino acid pool after salt stress. Proline accumulation occurs in plants mainly by de novo synthesis from glutamate. The last step of the proline biosynthetic pathway is catalyzed by pyrroline-5-carboxylate (P5C) reductase. A gene (*AT-P5C1*) encoding this enzyme in *A. thaliana* has been cloned and sequenced. Expression of *AT-P5C1* in *Escherichia coli* resulted in the complementation of a *proC* mutant to prototrophy. A comparison of the AT-P5C1 primary and secondary structures with those of six P5C reductases of other organisms is presented. With the exception of several functionally important amino acid residues, little conservation in the primary structure is seen; much greater similarity exists in the putative secondary structure. The AT-P5C1 protein is probably cytosolic. Under normal growth conditions, the P5C reductase mRNA level was significantly higher in roots and ripening seeds than in green tissue. A salt treatment of *A. thaliana* plants resulted in a 5-fold induction of the *AT-P5C1* transcript, suggesting osmoregulation of the *AT-P5C1* promoter region. Moreover, a time-course experiment indicated that this induction precedes proline accumulation.

Osmotic stress is a very common problem in agriculture. The most important osmotic stresses in agriculture are drought and high salinity. Free Pro accumulates in response to osmotic stress in a wide variety of organisms (Yancey et al., 1982): in protozoa (Kaneshiro et al., 1969; Poulin et al., 1987), eubacteria (Csonka and Hanson, 1991), marine invertebrates (Burton, 1991), algae (Schobert, 1977; Brown and Hellebust, 1978), and higher plants (reviewed by Aspinall and Paleg, 1981). Different roles have been proposed for Pro accumulation as an adaptive response; it has been suggested that Pro may function as an osmoticum (Wyn Jones et al., 1977), a sink of energy and reducing power (Blum and Ebercon, 1976), a nitrogen storage compound (Ahmad and Hellebust, 1988), a hydroxy-radical scavenger (Smirnoff and Cumbes, 1989), a compatible solute that protects enzymes (Schobert and Tschesche, 1978; Paleg et al., 1981, 1984), and a means of reducing the acidity (Venekamp et al., 1989). It may also play a role in the regulation of cellular redox potentials (Saradhi and Saradhi, 1991), as in animal cells (Phang, 1985). Whereas Pro accumulation is a primitive response to osmotic stress, conserved through evolution in both prokaryotes and eukaryotes, the sources of this accumulation seem to be different.

In eubacteria, Pro accumulation occurs via enhanced uptake. In enterobacteria, neither the synthesis nor the catabolism of Pro is subject to osmotic control (Csonka, 1988). These organisms depend on exogenous Pro for an osmoprotectant. Enhanced uptake occurs via de novo transcription of the Pro permease-encoding operons *proP* and *proU* (Csonka and Hanson, 1991). However, some data suggest that *Brevibacterium lactofermentum* (Kawahara et al., 1989, 1990) and *Bacillus subtilis* (Whatmore et al., 1990) could synthesize Pro under osmotic stress when Pro is not present in the environment.

In eukaryotes, and specifically in higher plants in which the problem is best documented, the main experimental approaches have been physiological. The mechanisms by which Pro accumulates are mainly de novo synthesis from glutamate (Morris et al., 1969; Boggess et al., 1976; Wang et al., 1982; Badzinski Buhl and Stewart, 1983; Rhodes et al., 1986; Venekamp and Koot, 1988; Venekamp et al., 1989) and, to a lesser extent, a stress-induced decrease in protein incorporation (Dungey and Davies, 1982), inhibition of Pro oxidation (Stewart et al., 1977), and enhanced proteolysis (Thompson et al., 1966). The Pro biosynthetic pathway is poorly known in higher plants and is thought to occur as in microorganisms, from glutamate via three enzymic steps.

P5C reductase (L-Pro:NAD[P]-5-oxido-reductase; EC 1.5.1.2) catalyzes the last step of the synthesis, the reduction of pyrroline-5-carboxylic acid to Pro. P5C reductase is the last enzyme in the Pro biosynthetic pathway in plants. This enzyme has been purified from *Hordeum vulgare* (Krueger et al., 1986), *Nicotiana tabacum* (LaRosa et al., 1991), and *Glycine max* (Chilson et al., 1991). Studies in higher plants have focused on the role played by P5C reductase in osmoregulation. An enhancement of its activity has been found in salt-stressed *Pennisetum typhoides* (Huber, 1974), *Mesembryanthemum nodiflorum* (Treichel, 1986), and *Chlorella autotrophica* (Laliberte and Hellebust, 1989) and in water-stressed barley (Argandona and Pahlich, 1991). Even if P5C reductase does not catalyze the rate-limiting step of the Pro biosynthetic pathway (LaRosa et al., 1991), its activity is osmoregulated concomitantly with Pro accumulation. The plant cDNA-encoding P5C reductase has been cloned from soybean nodules

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Abbreviations: ATCC, American Type Culture Collection; CHX, cycloheximide; COR, cordycepin; CRE, cAMP-regulated enhancer; P5C, pyrroline-5-carboxylate.

(Delauney and Verma, 1990). After osmotic stress, the P5C reductase mRNA level was shown to increase, suggesting that transcription of the P5C reductase gene might be osmoregulated (Delauney and Verma, 1990). In *Arabidopsis thaliana*, genes and enzymes involved in the Pro biosynthesis pathway are not known, despite their role in osmoregulation.

In this paper, we describe the cloning of a P5C reductase gene (*AT-P5C1*) and the corresponding cDNA in *A. thaliana* and the expression pattern of *AT-P5C1* in normal and stressed plant tissues.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg erecta or Bensheim were surface sterilized by dipping in 5% calcium hypochlorite for 10 min, followed by rinsing several times in water. Seeds were put on modified Murashige and Skoog medium (K1 medium) (Murashige and Skoog, 1962) containing Murashige and Skoog salts and vitamins, 1% Suc, and 0.8% agar (Difco) in Petri dishes. Growth took place in a culture chamber (16-h photoperiod, 21°C, 50% RH).

Stress Treatments

Salt induction experiments were performed with 10-d-old plantlets, which were taken from the solid agar medium and put in liquid culture medium containing 0, 0.5%, and 1% NaCl (0, 86, and 171 mM). At that stage of root development, plants can be taken out of the agar without wounding. After incubation, samples were briefly dried on Kleenex paper and stored at −70°C before analysis.

Measurement of Water Content

To determine the water content, plant samples were weighed and dried at 80°C during 24 h. The weight difference was considered to be the water content, expressed as percentage of fresh weight (=100%).

Amino Acid Extract and Analysis

Plantlets were analyzed by homogenizing 100 to 200 mg of material with a mixture of methanol:chloroform:water (12:5:2) (Bieleski and Turner, 1966). Chl was recovered by adding 2 volumes of chloroform and 1 volume of water to the extract. The aqueous layer was taken and completely evaporated. The residue was redissolved in 6 N HCl and hydrolyzed for 2 h under vacuum at 110°C. After evaporation at 85°C, the extracts were subsequently resuspended in the loading buffer (0.2 M Na-citrate, pH 2.2) and analyzed in an amino acid analyzer (Biotronik LC 5001).

Isolation of *AT-P5C1* Genomic Clones

Genomic DNA of *A. thaliana* ecotype Landsberg was extracted according to the method of Dellaporta et al. (1983). For the first polymerase chain reaction amplification, 200 ng was used in a 50-μL mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, all four deoxynucleotide triphosphates (0.2 mM each), 150 ng of each primer, and 2.5 units of *Taq* polymerase. The primers were 5′-GACTGCAG-TIATGCCIAAYACICCIGC and 5′-AGCTGCAGIAATIG-TIGTICCICCIGG, where Y is C or T, and I is inosine. *Pst*I sites are underlined. Thirty-five cycles at 94°C for 30 s, 46°C for 30 s, and 72°C for 60 s and a final extension for 5 min were performed. Because the yield was not sufficient, 10% of the volume was reamplified during 30 cycles under the same conditions as mentioned above and run on gel (1% low-melting point agarose). Two fragments of 320 and 730 bp were amplified and eluted from the gel using the GeneClean kit (Qiagen), digested with *Pst*I, and subcloned into pUC19. Clones were sequenced by the dideoxynucleotide method of Sanger et al. (1977). Only the 730-bp-long fragment contained significant similarity with known deduced P5C reductase sequences, and it was labeled by random priming and used to screen both a genomic and a cDNA bank. Thirty thousand clones (five genome equivalents) from an *A. thaliana* ecotype Landsberg erecta genomic bank in vector λ Charon 35 (a kind gift of Dr. D. Jofuku) were screened according to the method of Maniatis et al. (1982). Fifty thousand clones from an *A. thaliana* ecotype Landsberg erecta cDNA bank in vector λgt11 (purchased from Clontech) were screened according to the Amersham protocol. Hybridization and washes were carried out at 65°C.

Subcloning and DNA Sequence Analysis

cDNA inserts were subcloned as *Eco*RI fragments in pUC19. The two longest inserts gave rise to the pcP5CR5 and pcP5CR9 plasmids. Two genomic fragments, a 5.5-kb *Bgl*II fragment containing the entire coding sequence of *AT-P5C1* and a 4.6-kb *Eco*RI fragment, were subcloned in pUC19 and sequenced with internal primers (Sanger et al., 1977).

Genomic DNA Analysis

Preparation of total DNA was as described by Dellaporta et al. (1983), followed by a CsCl gradient. DNA (2 μg) was digested and separated on a 0.9% agarose gel. The DNA gel was blotted onto Hybond-N (Amersham), and the probe was labeled by random priming according to the manufacturer's protocol. The probe used was a 0.8-kb *Hae*III-*Pst*I fragment of the pcP5CR5 plasmid. Hybridization was carried out at 55°C in 5× SSC, 0.5% SDS, 5× Denhardt's solution (1× SSC is 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.0; 1× Denhardt's solution is 0.02% BSA, 0.02% Ficoll, 0.02% PVP).

mRNA Analysis

Total RNA was isolated from roots, stems, leaves, seeds, and flowers of in vitro-grown plants as described by Maniatis et al. (1982). RNA (12 μg) was electrophoresed on 1.5% agarose gels containing formaldehyde (6%) and transferred in 20× SSPE (3.6 M NaCl, 0.2 M $NaPO_4$, 0.02 M Na_2-EDTA, pH 7.7) to Hybond-N membranes (Amersham). The RNA gel blot was hybridized at 42°C for 16 h in 5× SSPE, 0.5% SDS, 50% (v:v) formamide, 5× Denhardt's solution. The washes were at 42°C in 2× SSPE, 0.1% SDS, twice for 10 min, each followed by 30 min at 42°C in 1× SSPE, 0.1% SDS and 30

min at 42°C in 0.5× SSPE, 0.5% SDS. That equal quantities of mRNA were loaded was confirmed by hybridization with a 25S RNA probe (pBS[I]KS$^+$ plasmids containing rDNA; Bauwens et al., 1991).

Secondary Structure Prediction

Secondary structure predictions were based on four different methods: the GOR method (Garnier et al., 1978), the homolog method (Levin et al., 1986), the GGBSM method (Gascuel and Golmard, 1988), and the method of Rooman and Wodak (1988). The methods were applied for each protein separately; a residue was considered likely to adopt a helical, an extended strand, or a coil conformation when at least three methods were in agreement.

Complementation Experiment

The *Escherichia coli proC* mutant ATCC No. 33475 F$^-$ *thi proC leu trp entA* (Wayne et al., 1976; *E. coli* Genetic Stock Center) was used for the complementation experiment. ATCC No. 33475 was transformed by electroporation with 10 ng of DNA of pcP5CR5, pcP5CR9, or, as control, pUC19. After electroporation, 1 mL of SOC medium (Maniatis et al., 1982) was added, and the cells were incubated at 37°C for 1 h, pelleted at 500*g* for 5 min, resuspended in 200 μL of M9 minimal medium (Maniatis et al., 1982), and plated on solid M9 minimal medium containing 100 μg mL^{-1} of ampicillin, 5 mM Leu, and 5 mM Trp. The efficiency of the electroporation was estimated to be about 4% by counting colonies on mineral medium that also contained 5 mM Pro.

Primer Extension

Primer extension was performed mainly as described by Ausubel et al. (1987). A 36-nucleotide primer in the first exon was hybridized against total *Arabidopsis* RNA (100 ng of primer against 50 μg of RNA). The primer itself was not radioactively labeled, but α-^{35}S-dATP was added in the extension reaction.

RESULTS

Pro Accumulation in *A. thaliana* during Salt Stress

Seeds of *A. thaliana* var Bensheim were germinated in the presence of increasing concentrations of NaCl (0–1%). Growth rate and free Pro content were analyzed 10 d after sowing (Fig. 1A). Most seeds did not germinate in the presence of 1% NaCl; those that did germinate did not grow. Free Pro content increased with the severity of the stress. A concentration of 0.5% NaCl was used in all further salt stress experiments because (a) there is a 6-fold increase in the Pro content, (b) the plant is stressed but can still grow and complete its life cycle, and (c) the water content of the plant is still high.

Figure 1B shows the time course of Pro accumulation for 10-d-old plantlets grown previously in K1 medium and subjected to a 0.5% NaCl stress. Pro begins to accumulate significantly after 4 h and reaches a plateau of 13% of the total free amino acid pool after about 24 h.

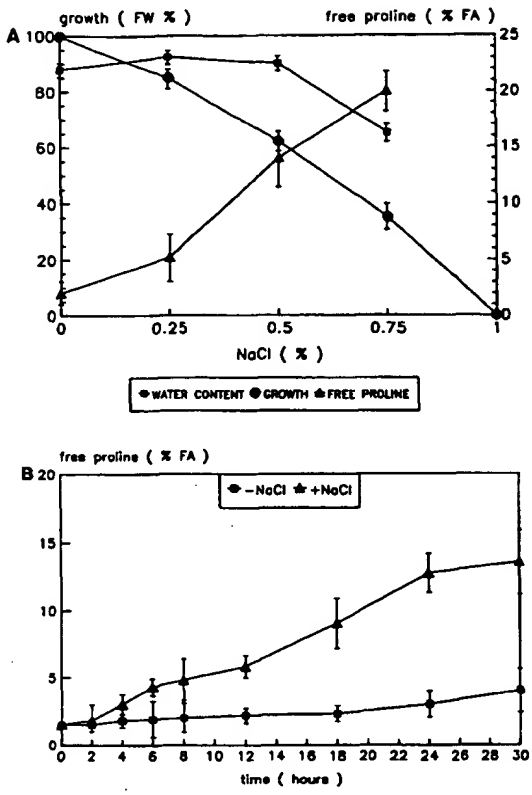

Figure 1. A, Water content (■), growth (●), and free Pro content (▲) in 2-week-old *A. thaliana* plantlets sown in K1 medium containing 0, 0.25, 0.5, or 1% (0, 43, 86, or 171 mM) NaCl. The growth is expressed as percentage fresh weight (FW %) versus the growth without NaCl (=100%); free Pro is expressed as percentage of the total free amino acid pool (% FA). B, Time course of Pro accumulation in 10-d-old *A. thaliana* plantlets in response to incubation in K1 liquid medium supplemented with 0.5% (w/v) NaCl (86 mM) (▲) and without NaCl (■). Values reported are the averages of three replicates each and are expressed as percentage of the total free amino acid pool (% FA). Samples contain about 20,000 nmol of free amino acid g^{-1} fresh weight.

De Novo Transcription and Translation Are Required for Pro Accumulation during Salt Stress

To measure the influence of de novo transcription and translation on Pro accumulation, an experiment was performed with the translation inhibitor CHX and the transcription inhibitor COR. Ten-day-old plantlets were incubated in liquid medium. At time zero, 0.5% NaCl was added to the incubation medium. At times −1, +2, +4, +6, and +12 h, CHX or COR was added in a concentration of 10 μg mL^{-1} or 5 mM, respectively. At time 24 h, free Pro concentration was measured (Fig. 2). Plants incubated in medium in which CHX or COR was added at times −1 and +2 h did not accumulate Pro, whereas all other plants did. COR influenced Pro accumulation even when added after 12 h of incubation, whereas CHX did not. This might be due to secondary effects of the inhibition. This experiment proves that de novo transcription and translation are indispensable during the first 4 h of salt stress before Pro begins to accumulate.

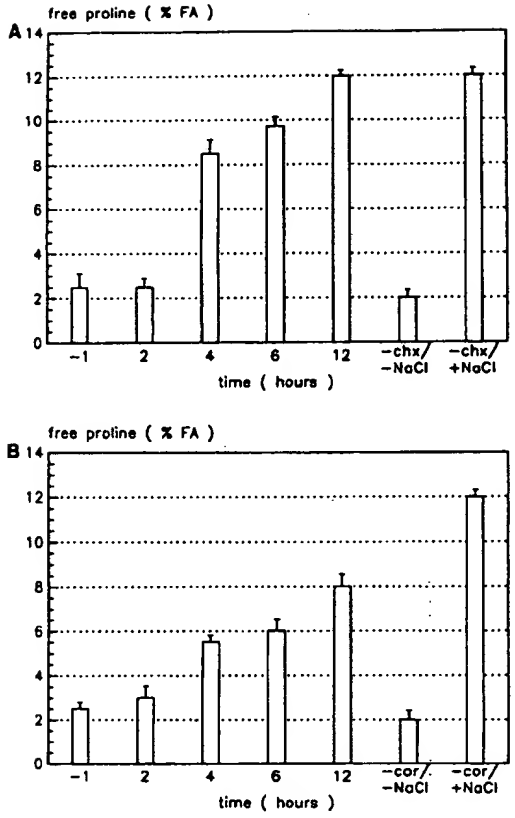

Figure 2. Effect of CHX (10 μg mL^{-1}) (A) and COR (5 mM) (B) on the salt-induced Pro accumulation. At time −1 h, 10-d-old *A. thaliana* plantlets were incubated in liquid medium. NaCl was added at time zero. Inhibitors were added at the time shown on the abscissa. At time 24 h, the samples were harvested and free Pro content was determined. Values reported are the averages of three replicates each and are expressed percentage of the total free amino acid pool (FA %). Controls are samples incubated without inhibitor and without salt (−chx/−NaCl; −cor/−NaCl) and samples incubated in the presence of salt but without inhibitor (−chx/+NaCl; −cor/+NaCl).

Isolation and Characterization of an *Arabidopsis* P5C Reductase Gene

To investigate further whether Pro accumulation is a consequence of de novo transcription of genes in the biosynthetic pathway, *AT-P5C1*, a P5C reductase gene in *A. thaliana*, was cloned. Degenerate primers were chosen from amino acid stretches that were conserved among the deduced P5C reductase sequences of *E. coli* (Deutch et al., 1982), soybean (Delauney and Verma, 1990), and yeast (M.C. Brandriss, GenBank accession No. M57886). The first primer was based on the peptide sequence VMPNTP, and the second was based on the sequence PGGTTI. A polymerase chain reaction (see "Materials and Methods") was performed with genomic DNA of *A. thaliana*. A 730-bp-long amplified fragment, designated P5CR730, was used to screen libraries.

An *A. thaliana* λgt11 cDNA bank was screened, and, from 50,000 recombinant clones, 12 positive clones were obtained. The largest inserts (1.1 and 0.9 kb) were cloned in pUC19 to give rise to the plasmids pcP5CR5 and pcP5CR9, respectively. Their sequences were identical and overlapping: the integrated 1.25-kb sequence is shown in Figure 3A. The pcP5CR5 insert sequence appears to be full length at the 5′ end and extends 68 bp beyond the putative termination codon (position 2528). The pcP5CR9 insert sequence extends 272 bp beyond the putative stop codon, has a short (19 bp) poly(A) tail, and starts 104 bp after the putative start codon (position 1102). Both cDNA 3′ ends are downstream from potential polyadenylation sites (Dean et al., 1986) (at positions 2578 and 2730; underlined in Fig. 3A). The 5′ end of the mRNA was defined by primer extension. The primer used was at position 1296 to 1331. The most abundant primer extension product suggests a start of transcription at position −127 with respect to the first AUG of the mRNA (data not shown). This is in agreement with the start of the pcP5CR5 insert (position 975 in Fig. 3A). The 1.25-kb *AT-P5C1* cDNA sequence contains an open reading frame encoding a polypeptide of 28,626 D (276 amino acids) with a calculated isoelectric point of 8.64.

To determine the structure of the *AT-P5C1* gene, an *A. thaliana* ecotype Landsberg erecta λ Charon 35 genomic library was screened with P5CR730 as the probe. From 30,000 recombinant clones (five genome equivalents), 24 hybridizing plaques were obtained; 6 were further analyzed and had an insert greater than 16 kb. From 2 of these, a 5.5-kb *Bgl*II fragment (designated gP5CR23) and an overlapping 4.6-kb *Eco*RI fragment (designated gP5CR22) were sequenced, covering an 8-kb region (Fig. 3B). The 3.1-kb sequence presented (Fig. 3A) covers 1101 bp of the 5′ untranslated region and 570 bp at the 3′ end. The sequences of the pcP5CR5 and pcP5CR9 inserts fitted perfectly with the deduced exon sequences of the genomic *AT-P5C1* sequence.

The intron/exon structure is presented in Figure 3B. *AT-P5C1* contains six short introns (81, 93, 125, 115, 78, and 106 bp). They all contain the GT and AG dinucleotide consensus at their respective 5′ and 3′ ends (Csank et al., 1990).

The upstream region of *AT-P5C1* shows several interesting features: a palindromic sequence (positions 722–742 in Fig. 3A) and a 26-bp element

$$(AG^{C}_{T}A^{G}_{T}CTCG^{T}_{C}CAGC^{TCA}_{CGG}CCCGTCTGA)$$

repeated seven times between the positions 912 and 1089 (underlined in Fig. 3A). In the last repeat, 4 bp are truncated. No significant similarity between the sequence of the repeat and sequences in data banks has been found. Sequences identical with the human cAMP-regulated enhancer sites (CRE-1), namely 5′-CGTCA-3′ (Fink et al., 1988), were found six times, at positions 675, 810, 919, 971, 997, and 1049 (Fig. 3A). A 6-bp sequence identical with the GCN4-binding site core 5′-TGACTA-3′ (Arndt and Fink, 1986) is also present upstream from the transcription initiation site, at position 500 (Fig. 3A).

Homology with Other P5C Reductase Genes

Homology of the *AT-P5C1* cDNA with all known P5C reductase cDNA sequences (*E. coli*, soybean, human, yeast, *Methanobrevibacter smithii*, *Pseudomonas*) is presented in

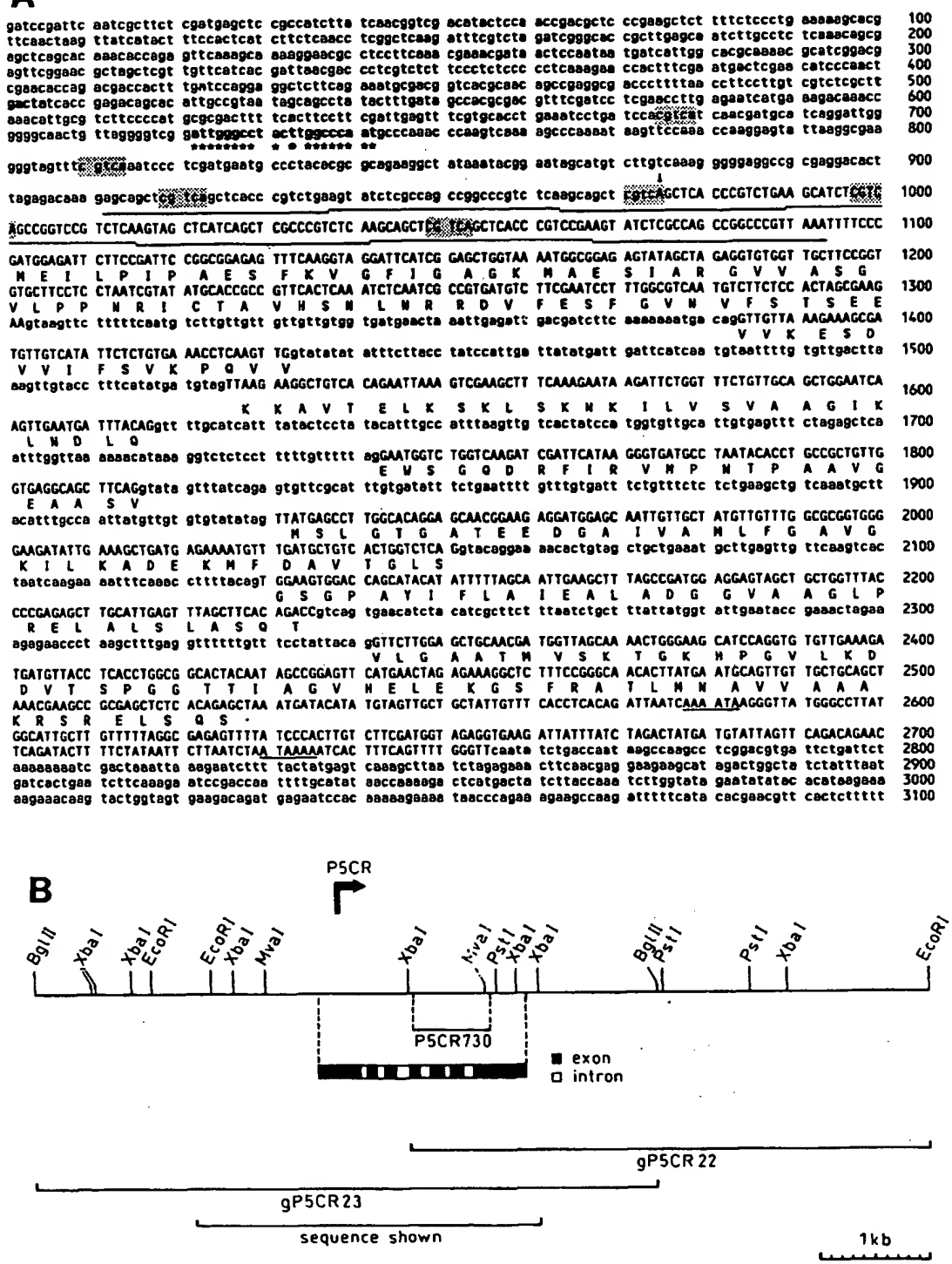

Figure 3. A, Nucleotide sequence of *AT-P5C1* and surrounding 5′ and 3′ regions. The deduced exon sequences are shown in uppercase letters and correspond to the cDNA sequences of pcPC5CR5 (975–2596) and pcP5CR9 (1206–2755). The deduced amino acid sequence is indicated below the DNA sequence. CRE-1 motifs are shaded, the palindrome is in boldface with asterisks, and the GCN4 motif is in boldface (500–505). The putative transcription start (975) is indicated by an arrow. The repeated sequences and the putative polyadenylation signal sequences are underlined. B, Genomic organization of the *AT-P5C1* and flanking regions (8 kb in total). gP5CR22 and gP5CR23 are the two overlapping genomic clones that have been sequenced. The *Eco*RI site present at the 3′ sequence is apparently a vector-derived cloning site as shown by the Southern blot analysis (Fig. 4).

Table I. The *B. subtilis proC* sequence (Lewis and Wake, 1989) is not included in the table because it is only partially known. The *Arabidopsis* and soybean sequences are 71.3% identical. No comparison between genomic eukaryotic sequences could be made, because the *A. thaliana* genomic sequence is the only one available.

Genomic DNA Analysis

To determine the sequences related to *AT-P5C1* in the *Arabidopsis* genome, total DNA (2 μg) was digested with five different restriction enzymes (*Bgl*II, *Eco*RI, *Hae*III, *Nhe*I, and *Xba*I) and subjected to Southern blot analysis using only the translated coding sequence of *AT-P5C1* (0.8 kb) as a probe. With the exception of *Xba*I, none of the enzymes chosen cut in *AT-P5C1*. After overnight hybridization at 55°C, the blot was washed at medium (2× SSC, 0.1% SDS; 55°C) or high (0.1× SSC, 0.1% SDS; 65°C) stringency. At medium stringency, two types of bands can be seen (Fig. 4A): the strongly hybridizing bands that correspond to the structure of *AT-P5C1* and less intense bands that almost all disappear when the blot is washed at high stringency (Fig. 4B).

Predicted Primary and Secondary Structure of P5C Reductase

Deduced P5C reductase amino acid sequences from different organisms are aligned in Figure 5; the degree of similarity between these sequences is presented in Table I. Enzymic studies indicate that P5C reductase can use either NADH or NADPH as cofactor (Miler and Stewart, 1976; Rossi et al., 1977; Costilow and Cooper, 1978; Krueger et al., 1986; Rayapati et al., 1989). Plant P5C reductase has been reported to have a lower K_m for NADPH than for NADH. The keys to search NADH- and NADPH-binding sites were extracted from Branden and Tooze (1991) and from Hanukoglu and Gutfinger (1989), respectively. There was no NAD(P)H-binding site consensus that fitted all P5C reductases. Plant and human P5C reductase fitted the consensus for the NADPH-binding site, whereas the key for NADH-binding site fitted with the *Saccharomyces cerevisiae* and *M. smithii* sequences. For other reductases, one (for *Pseudomonas*) and two (for *E. coli*) mismatches with the NADPH key were found. The deduced AT-P5C1 amino acid sequence is very close to that

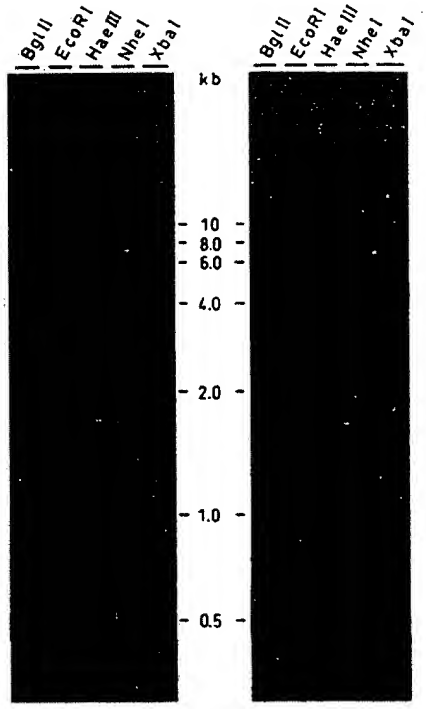

Figure 4. Genomic DNA gel blot analysis of *A. thaliana*. Total DNA (2 μg) was digested with *Bgl*II, *Eco*RI, *Hae*III, *Nhe*I, and *Xba*I. Hybridization was done overnight with the translated coding region of *AT-P5C1* (see "Materials and Methods"). A, Wash performed in 2× SSC, 0.1% SDS at 55°C. Exposure was overnight. B, Wash performed in 0.1× SSC, 0.1% SDS at 65°C. Exposure was for 40 h.

of soybean (77.7% similarity). Plant P5C reductases have a lower similarity when compared to yeast (30.1 and 29.9%) than to human (42 and 43.1%) or to *E. coli* (40.2 and 37.2%) when the sequences are aligned by the addition of appropriate gaps; plant P5C reductases share no significant similarity with the *M. smithii* sequence, which is also very far from the other species. The two bacterial sequences display poor similarity (36.8%), although *proC* is a housekeeping gene (Savioz et al., 1990).

The predicted secondary structures of the seven known P5C reductase sequences are very similar (Fig. 5). They

Table I. *Identity (%) of the nucleic acid (NA) and deduced amino acid (AA) sequences of AT-P5C1 with other P5C reductase cDNAs according to PC/GENE*

A, *A. thaliana*; E, *E. coli* (Deutch et al., 1982); H, *H. sapiens* (Dougherty et al., 1992); M, *M. smithii* (Hamilton and Reeve, 1985); P, *P. aeruginosa* (Savioz et al., 1990); S, *G. max* (Delauney and Verma, 1990); Y, *S. cerevisiae* (M. C. Brandriss, GenBank accession No. M57886).

	E		H		M		P		S		Y	
	NA	AA	NA	AA	NA	AA	NA	AA	NA	AA	NA	AA
A	56.9	40.5	55.2	42.0	52.8	22.0	50.1	36.6	71.3	77.7	51.7	30.1
E			57.3	36.4	49.9	22.0	54.0	36.8	54.2	37.2	51.9	27.9
H					53.3	21.0	62.4	38.5	57.2	43.1	51.0	31.0
M							44.6	23.0	48.1	23.0	58.5	23.0
P									54.9	38.5	49.8	23.8
S											51.6	29.9

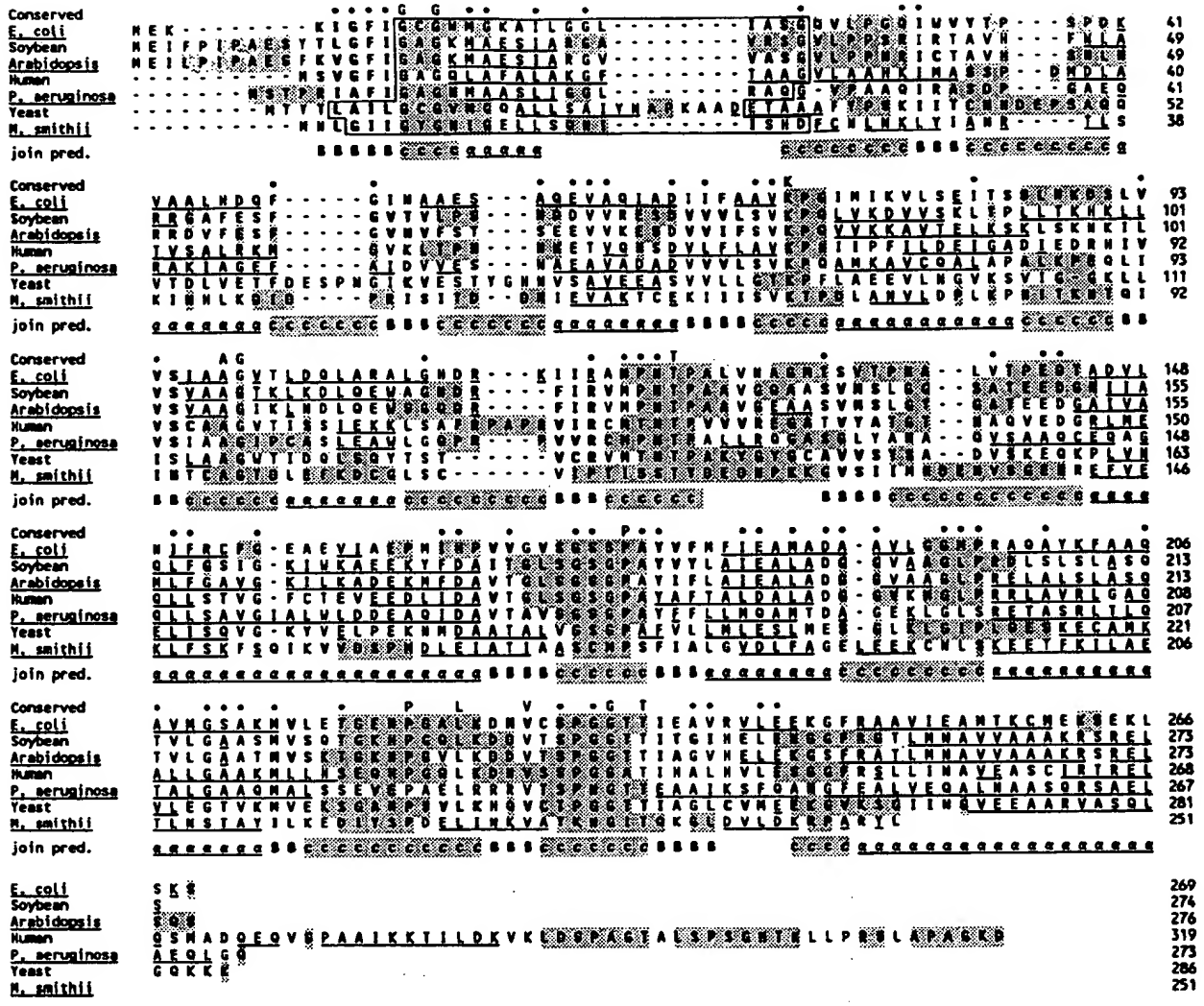

Figure 5. Comparison of the deduced P5C reductase amino acid sequence of AT-P5C1 with that of *E. coli* (Deutch et al., 1982), soybean (Delauney and Verma, 1990), human (Dougherty et al., 1992), *P. aeruginosa* (Savioz et al., 1990), *S. cerevisiae* (M.C. Brandriss; GenBank No. M57886), *M. smithii* (Hamilton and Reeve, 1985). Alignment was done using the Myers and Miller (1988) alignment algorithm program (PC/GENE). The conserved sequence is indicated at the top. Similar amino acids are indicated with dots (PC/GENE Program). NAD(P)H-binding sites are boxed. The predicted secondary structure is presented as follows: residues in an α-helix (α) are underlined, residues in a β-strand (β) are in boldface, and residues in coil structures (c) are shown against a stippled background. All others are not assigned.

feature an alternation of α-helices and β-strands (separated by coil regions). This organization is compatible with the existence of one or more β-sheet(s) surrounded by α-helices (J. Richelle and S. Wodak, unpublished data). Among the 12 amino acids conserved in all sequences (=4%) (Fig. 5), 11 residues (4 glycyl, 1 lysyl, 1 alanyl, 1 leucyl, 2 threonyl, and 2 prolyl) are present in the predicted coil structure.

Expression of *AT-P5C1* in *E. coli*

An *E. coli* Pro auxotroph, ATCC No. 33475, that lacks a functional P5C reductase was electroporated with two pUC19 plasmid derivatives, pcP5CR5 and pcP5CR9, containing *Arabidopsis* P5C reductase cDNA inserts (see "Materials and Methods"). Expression of the inserts was under the control of the *lacZ* promoter. This complementation was carried out with 10 ng of the pcP5CR5 plasmid and the pcP5CR9 plasmid, which lacks the putative NADPH site; pUC19 was used as a negative control. The pcP5CR5 cDNA sequence, but not that of pcP5CR9, complemented ATCC No. 33475 (Fig. 6). This proves that *AT-P5C1* encodes a functional P5C reductase.

Tissue-Specific Expression of *AT-P5C1*

AT-P5C1 expression was investigated by RNA gel blot analysis in different tissues of plants grown under normal conditions or with 2 mM Pro. Total RNA from seed, flower,

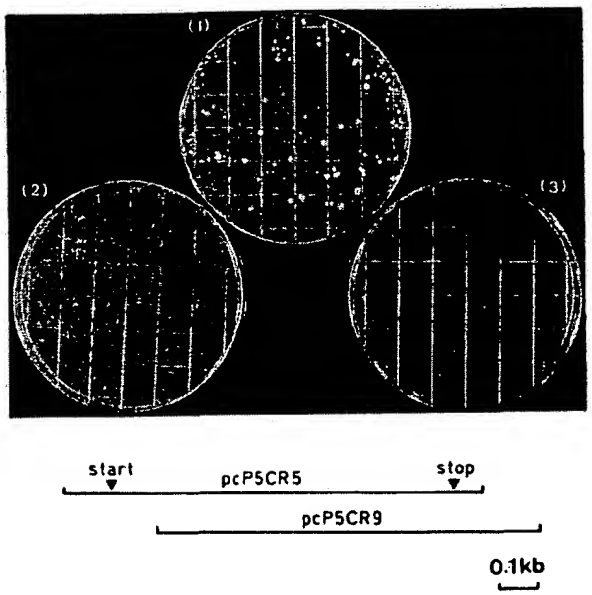

Figure 6. Complementation of *E. coli* ATCC 33475, a *proC* Pro auxotroph, with *AT-P5C1* cDNA to prototrophy. ATCC 33475 cells electroporated with 10 ng of pcP5CR5 (1), 10 ng of pUC19 (2), and 10 ng of pcP5CR9 (3) and plated on minimal media containing ampicillin, Leu, and Trp. Start and stop indicate translation signals.

root, stem, and leaf was probed with the *AT-P5C1* cDNA (Fig. 7A). Under stringent conditions, only one band (1.4 kb) was visible. In plants grown under normal conditions, a decreasing expression is found in the following order: seeds > roots > flowers > stems = leaves (Fig. 7A). The expression pattern shown in Figure 7A is only that of *AT-P5C1*. Under stringent conditions, the pattern of expression is the same using the entire coding sequence or the 3′ trailer, which is *AT-P5C1* specific (data not shown). In plants grown in the presence of Pro, *AT-P5C1* expression is not repressed as in *E. coli* (Rossi et al., 1977) (Fig. 7A).

Expression of *AT-P5C1* under Salt Stress

Figure 7B shows *AT-P5C1* expression during the first 4 h of salt stress. Ten-day-old plants, previously grown in K1 medium, were incubated for different times in K1 supplemented with 0.5% NaCl. The level of *AT-P5C1* transcripts already started to increase after 15 min of incubation; 3 h after the beginning of salt stress, the amount was about the same as in total RNA of 10-d-old plants grown in K1 medium supplemented with 0.5% NaCl.

An RNA gel blot analysis was performed with RNA from 3-week-old plants sown in K1 mineral medium with or without 0.5% NaCl. *AT-P5C1* cDNA (pcP5CR5 *Eco*RI insert) was used as probe. In the presence of 0.5% NaCl, the RNA abundance in leaves and roots increased 5- and 2-fold, respectively (Fig. 7C).

DISCUSSION

Free Pro accumulation is a widely observed response to osmotic stress. In *Arabidopsis*, accumulation of Pro occurs after salt stress (NaCl) and can reach 20% of the total free amino acid pool (in the presence of 0.75% NaCl) (Fig. 1A). Whatever the precise role(s) of Pro during osmotic stress, it must be important or otherwise natural selection would have occurred against such an energy-intensive process. Considering Pro accumulation merely as a symptom of stress would be an oversimplification.

In plants, Pro accumulation has been reported to be due primarily to de novo synthesis from glutamate. Experiments using CHX and COR show that de novo transcription and translation are indispensable in the first 4 h of the stress for Pro accumulation to occur (Fig. 2). To determine to what extent salt-induced transcription of Pro biosynthetic enzyme-encoding genes could be involved in Pro accumulation in *Arabidopsis*, *AT-P5C1* was cloned; it encodes a functional enzyme, since upon expression in *E. coli*, *AT-P5C1* cDNA complemented a *proC* mutant (Fig. 6).

The 5′ untranslated region of *AT-P5C1* shows several

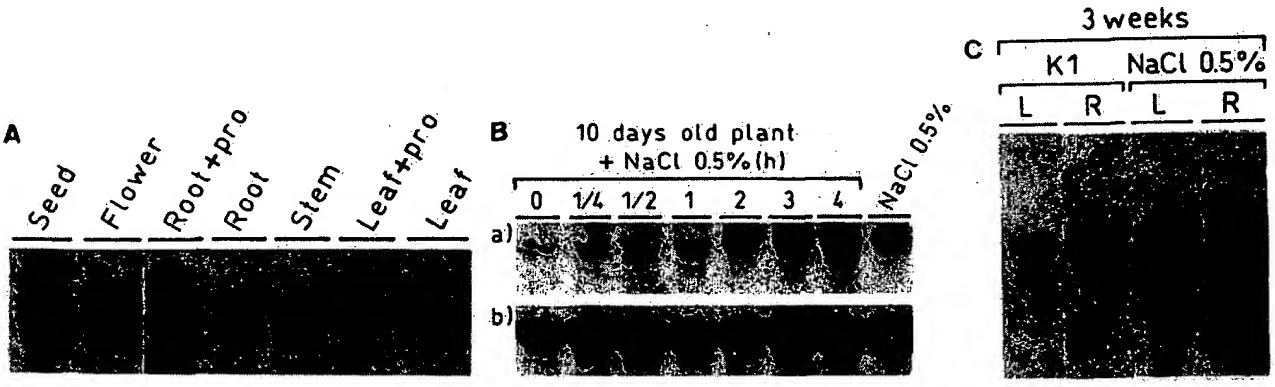

Figure 7. RNA gel blot analysis. Exposure was overnight with flash-sensitized film. The *AT-P5C1* cDNA was used as a probe. A, RNA was extracted from ripening seeds and flowers of mature plants, root, stem, and leaves of 3-week-old plants grown without or with 2 mM Pro (+PRO). B, RNA was extracted from 10-d-old plantlets grown on K1 medium and then incubated for varying times (h) in liquid K1 medium supplemented with 0.5% NaCl or from plantlets in K1 + 0.5% NaCl (0.5% NaCl) for 10 d and hybridized with *AT-P5C1* cDNA probe (a) or 25S RNA probe (b). C, RNA was extracted from 3-week-old plants grown on K1 mineral medium without and with 0.5% NaCl. L, Leaf; R, root.

potential regulatory sites (Fig. 3A): a palindromic sequence, a GCN4-analogous binding site motif, and a 26-bp element repeated seven times. Sequences identical to the human CRE-1 sites have also been found six times in the 5′ untranslated *AT-P5C1* region. Further experiments are needed to investigate their possible role in the regulation of *AT-P5C1* expression.

Genomic hybridization suggested the existence of different *AT-P5C1*-related genes (Fig. 4A), none of which is very closely related to *AT-P5C1* (Fig. 4B). Most of the amino acid biosynthetic enzymes examined are encoded by multiple genes (reviewed by Coruzzi, 1991). The AT-P5C1 protein is most probably cytosolic: the N terminus is very similar to that of prokaryotic *proC* sequences and the yeast N-terminal *PRO3* sequence (Fig. 5); there is no structural similarity with the consensus chloroplast target sequence (von Heijne et al., 1989). However, the existence of P5C reductase isoenzymes has recently been demonstrated in soybean (Szoke et al., 1992).

A comparison of known P5C reductase sequences at the nucleic acid and deduced amino acid levels revealed interesting features. Except between *A. thaliana* and soybean, P5C reductase sequences show less identity at the amino acid level than at the nucleic acid level (Table I). Only some (4%) functionally or structurally important sites are conserved among the seven deduced P5C reductase amino acid sequences and are present in predicted coil structures. All of the secondary structure predictions for the different P5C reductases are β-sheet(s) surrounded by α-helices, which are thermodynamically stable structures (Fig. 5).

The expression of *AT-P5C1* was studied by RNA gel blot analysis in different tissues and under salt stress. The expression is complex and is regulated at different levels: *AT-P5C1* is differentially expressed in organs and with the life cycle (data not shown) and can be induced by salt treatment.

AT-P5C1 transcripts are more abundant in roots compared to leaves, although free Pro content is higher in the leaves compared to roots (Fig. 7A; *A. thaliana*, N. Verbruggen, unpublished data; sunflower, Golan-Goldhirsch et al., 1990). This apparent contradiction between the amount of *AT-P5C1* transcript and the free Pro content can have different explanations; for example, it may be that AT-P5C1 is not the only P5C reductase in the leaves or that chloroplastic P5C reductase is the more active one, as in pea leaves (Rayapati et al., 1989). Posttranscriptional regulation or a mechanism to transport Pro from roots to leaves could also be postulated.

The amount of *AT-P5C1* mRNAs is higher in flowers than in leaves (Fig. 7A) and free Pro accumulates more in flowers than in leaves of flowering plants (5.2% of the total free amino acid pool versus 0.5%) (N. Verbruggen, unpublished data). These observations support the idea that Pro is likely to play a role in flower development, as suggested by Vansuyt et al. (1979), Mutters et al. (1989), and Walton et al. (1991). Furthermore, *AT-P5C1* transcripts are very abundant in ripening seeds, which have to face drought stress. This corroborates with the high free Pro content found in *A. thaliana* (8% of the total amino acid pool) (N. Verbruggen, unpublished data) and in *Vicia faba* seeds (Venekamp and Koot, 1988).

AT-P5C1 is expressed more in salt-stressed *Arabidopsis* plants (Fig. 7, B and C). Induction of *AT-P5C1* transcript is already visible 15 min after the beginning of salt stress (Fig. 7B), and the increase in the amount of *AT-P5C1* transcript levels off after 3 h (Fig. 7B). It takes more time for Pro accumulation to occur (between 2 and 4 h) in salt-stressed (0.5% NaCl) *A. thaliana* plantlets (Fig. 1B). This *AT-P5C1* mRNA increase is more pronounced in leaves than in roots (Fig. 7C). Free Pro accumulates more in the leaves, where it serves as cytosolic osmotic solute. Under normal growth conditions, roots already have to face osmotic problems; therefore, we can imagine that the salt induction of cytosolic P5C reductase mRNA is higher in leaves.

The transcription of *AT-P5C1* should be studied under other stress situations in which Pro has been reported to accumulate: cold (Havaux and Lannoye, 1982), gas pollution (Anbazhagan et al., 1988), nutrient deficiencies (Savitskaya, 1976), low pH (International Rice Research Institute, 1973), and the presence of heavy metals (Saradhi and Saradhi, 1991). According to the theory that Pro is an effective hydroxyl radical scavenger (Smirnoff and Cumbes, 1989), its de novo synthesis should be expected upon oxidative stress.

All of the physiological observations concerning Pro accumulation cannot be interpreted by the study of *AT-P5C1* alone. Further investigations will be carried out to study the other genes of the Pro biosynthetic pathway.

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Cloning Human Pyrroline-5-carboxylate Reductase cDNA by Complementation in *Saccharomyces cerevisiae**

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Pyrroline-5-carboxylate reductase (EC 1.5.1.2) catalyzes the NAD(P)H-dependent conversion of pyrroline-5-carboxylate to proline. We cloned a human pyrroline-5-carboxylate reductase cDNA by complementation of proline auxotrophy in a *Saccharomyces cerevisiae* mutant strain, DT1100. Using a HepG2 cDNA library in a yeast expression vector, we screened 10^5 transformants, two of which gained proline prototrophy. The plasmids in both contained similar 1.8-kilobase inserts, which when reintroduced into strain DT1100, conferred proline prototrophy. The pyrroline-5-carboxylate reductase activity in these prototrophs was 1–3% that of wild type yeast, in contrast to the activity in strain DT1100 which was undetectable. The 1810-base pair pyrroline-5-carboxylate reductase cDNA hybridizes to a 1.85-kilobase mRNA in samples from human cell lines and predicts a 319-amino acid, 33.4-kDa protein. The derived amino acid sequence is 32% identical with that of *S. cerevisiae*. By genomic DNA hybridization analysis, the human reductase appears to be encoded by a single copy gene which maps to chromosome 17.

Pyrroline-5-carboxylate (P5C)[1] reductase (EC 1.5.1.2) catalyzes the reduction of P5C to proline in an NAD(P)H-dependent reaction which is both the first committed and final step in proline synthesis. P5C reductase activity is present in the cytosol of virtually all mammalian tissues and cultured cells. In addition to its role in proline synthesis, P5C reductase, together with the other enzymes of P5C and proline metabolism, may influence the ratios of oxidized/reduced pyridine nucleotides (1).

Two lines of evidence suggest that there may be two (or more) forms of P5C reductase. First, kinetic studies show that P5C reductase from various tissues displays different cofactor preferences. P5C reductase from human erythrocytes (2), bovine retina (3), and rat lens (4) has a 20–60-fold lower K_m for NADPH *versus* NADH and has a 5–12-fold higher affinity for P5C with NADPH as cofactor (2). Purified erythrocyte P5C reductase utilizes NADPH exclusively when both pyridine nucleotides are present at physiologic concentrations (2). By contrast, P5C reductase from cultured human fibroblasts (5) and a lymphoblastoid cell line (LHN cells) (6) has a similar affinity for either cofactor and an affinity for P5C which is not dependent on the choice of cofactor. Second, the sensitivity of P5C reductase to inhibitors is tissue-specific. The enzyme from cultured human fibroblasts and LHN cells is inhibited by proline but not by $NADP^+$; the converse is true for P5C reductase from erythrocytes, lens, and retina (2). Based on these differences in kinetic characteristics and sensitivity to inhibitors, Phang and his colleagues (1, 2, 5–7) suggested that there are different forms of P5C reductase in various tissues. Furthermore, they propose that the reductase reaction serves different metabolic roles in these tissues (for review, see Ref. 1). In fibroblasts, cartilage, and other tissues with a high requirement for proline, the primary function of the reaction is to synthesize this amino acid (7). Alternatively, in erythrocytes, where the enzyme is inhibited by $NADP^+$ and there is no requirement for proline synthesis, the primary function of the reaction may be to produce $NADP^+$ necessary for activity of the hexose monophosphate shunt.

As an initial step in determining the molecular basis for the tissue-specific characteristics of P5C reductase and the factors which influence its functional roles, we set out to clone a human P5C reductase cDNA and ultimately the structural gene(s). At the time of institution of these studies, primary sequence information for P5C reductase was available only from microorganisms (8–10)[2] and soybean (11). Small amounts of P5C reductase had been purified from mammalian sources, but the quantity was insufficient for antibody production or sequence determination (2–4). Therefore, we elected to utilize a functional cloning strategy, namely complementation in a *Saccharomyces cerevisiae* mutant strain lacking the reductase. We reasoned that P5C reductase should be amenable to this cloning strategy. The human protein appears to be a homopolymer comprised of moderately sized (~30 kDa) subunits (2). Mutant strains of *S. cerevisiae* lacking P5C reductase activity have been well characterized (12) and are auxotrophic for proline, providing a selection system for complementation. Appropriate human cDNA libraries in a yeast expression vector recently have been described (13).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M77836.

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[1] The abbreviations used are: P5C, pyrroline 5-carboxylate; bp, base pair(s); LHN, lymphoblastoid, SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; HEK, human embryonic kidney; kb, kilobase(s).

[2] M. C. Brandriss and D. A. Falvey, submitted for publication.

Using similar complementation strategies, the human homolog for the cell cycle control gene (*cdc2*) (14), a CCAAT-binding transcription factor (15), and three multifunctional genes involved in *de novo* purine synthesis (13, 16) have been cloned.

In this report, we describe the isolation and characterization of human P5C reductase cDNA by complementation in *S. cerevisiae* and mapping of the human structural gene.

MATERIALS AND METHODS AND RESULTS[3]

DISCUSSION

By complementation of an *S. cerevisiae* P5C reductase mutant strain, we have cloned two independent cDNAs encoding human P5C reductase. Phenotypic complementation occurred despite the relatively low level of P5C reductase activity measured in the transformants (Table II). Either wild type *S. cerevisiae* have far more P5C reductase activity than required for growth on a medium lacking proline or the enzymatic activity measured *in vitro* does not accurately reflect that *in vivo*. Clearly, the low P5C reductase activity in the prototrophic transformant (KD100) does not result from a low level of P5C reductase mRNA (Fig. 5*A*). This lack of correlation between the levels of mRNA and enzymatic activity could be explained by inefficient translation of the message, lack of necessary post-translational processing, instability of the human protein in yeast, or a negative interaction of the truncated yeast P5C reductase subunits with the wild type human subunits. Antibodies against the human P5C reductase protein will be useful in distinguishing among these possibilities.

Our longest human P5C reductase cDNA is 1.8 kb in length and has 11 bp upstream of the first AUG. We propose that this first AUG marks the start of translation based on two lines of evidence. First, the context provides a reasonable Kozak consensus sequence (36). There is an A at position −3, a G at −6, and purine at +4 (Fig. 3). Second, use of the next downstream AUG would eliminate amino-terminal protein sequence which has homology with the reductases from other species (Fig. 4). Initiation of translation at the first AUG gives a 957-bp open reading frame encoding a protein of 319 amino acids with a predicted molecular mass of 33.4 kDa. This is consistent with the size of the P5C reductase monomer purified from human erythrocytes which was estimated to be ~30 kDa by SDS-polyacrylamide gel electrophoresis (2).

The predicted amino acid sequence for human P5C reductase has considerably homology to the reductases from microorganisms and soybean (Fig. 4). There are blocks of 7–14 amino acids in which the identity is >75%. Presumably, these regions are important for the structure and/or function of the enzyme. We were unable to identify with certainty an NADPH binding site that fits the consensus based on several reductase enzymes (I__G_GTGIAPF__F[~100 amino acids] Y_CG____M) (37). Perhaps this reflects the fact that P5C reductase can use either NADPH or NADH. Also, it is interesting that the human protein extends approximately 40 carboxyl terminal amino acids beyond any regions of identity with the reductase sequences from microorganisms and soybean.

P5C reductase mRNA is present in a wide variety of cell lines, including HepG2 cells, skin fibroblasts, HEK cells, HeLa cells, AD12 cells, and MCF 7 cells, in agreement with the presence of P5C reductase activity in virtually all mammalian cells and tissues (38). The high level of enzyme activity in HepG2 cells as compared to fibroblasts can be partially accounted for by the greater amount of reductase mRNA in the former. The poly(A$^+$) RNA from HepG2 cells exhibits at

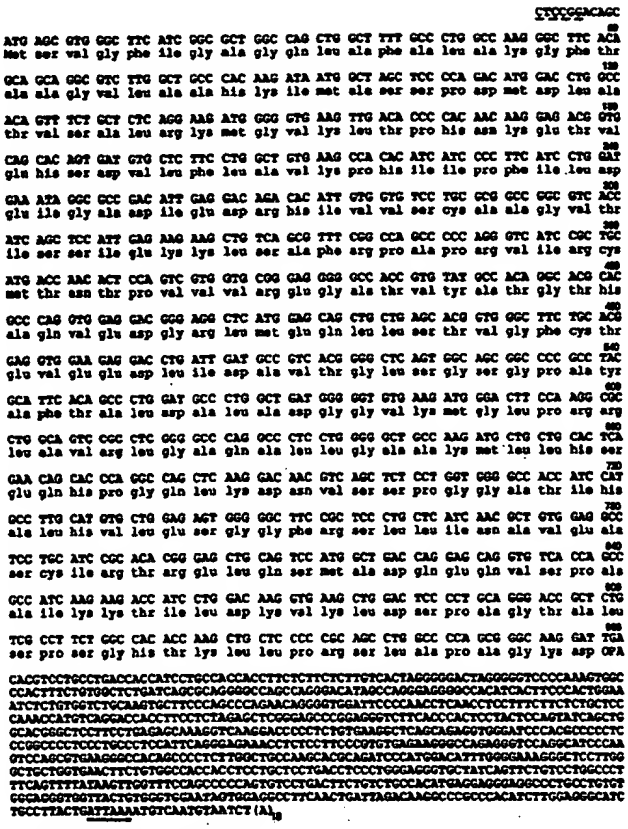

FIG. 3. **The nucleotide sequence of human P5C reductase cDNA.** The bases indicated by the *dashed underline* were determined from the sequence of huP5CR.2. The predicted amino acid sequence is shown below. The proposed polyadenylation signal is *underlined*. The nucleotide sequence is numbered from the A of the first ATG.

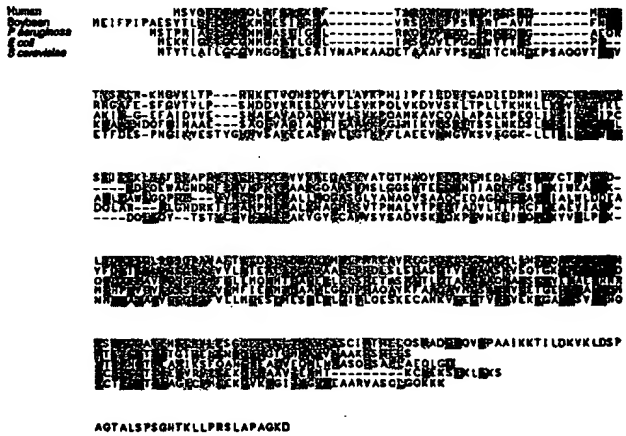

FIG. 4. **Comparison of the amino acid sequence of human P5C reductase with that of *Escherichia coli* (10), soybean (11), *Pseudomonas aeruginosa* (8), and *S. cerevisiae*.**[3] Amino acids identical with the human sequence are *shaded*. Alignment was done using the Needleman and Wunsch alignment algorithm program.

[3] Portions of this paper (including "Materials and Methods", "Results," Tables I and II, and Figs. 1, 2, 5, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

least two bands when probed with huP5CR.1 cDNA. These different species could be due to differential splicing of the P5C reductase mRNA which, if the pattern varied in a tissue-specific fashion, could explain the different enzymatic forms of the protein. Alternatively, the two P5C reductase transcripts could reflect the use of two sites for polyadenylation. In this regard, the proposed polyadenylation signal in both phuP5CR.1 and phuP5CR.2, AUUAAA, is not the canonical AAUAAA (39).

We examined the human genomic organization of the human P5C reductase gene by Southern blot analysis. The gene appears to be single copy with a relatively simple structure, as evidenced by the low number of hybridizing fragments even at low stringency (Fig. 6). If the tissue-specific differences in P5C reductase kinetics and inhibitor sensitivity were due to multiple P5C reductase genes, we would expect a more complex pattern of genomic fragments.

A human genetic disease caused by an abnormality of P5C reductase has not yet been recognized. The expected phenotypic features for deficiency of P5C reductase might include runting, chondrodysplasia (7), cataracts (1), impaired lactation (40), and/or hemolytic anemia (41). Results with two human/rodent hybrid cell mapping panels showed that the reductase gene mapped to human chromosome 17. Examination of the phenotypes of the human genetic diseases mapped to this chromosome (42) or to its major murine counterpart (mouse chromosome 11) (43) does not reveal obvious candidates.

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SUPPLEMENTAL MATERIAL TO: Cloning Human Pyrroline 5-Carboxylate Reductase cDNA by Complementation in *Saccharomyces cerevisiae*

Kristiann M. Dougherty, Marjorie C. Brandriss and David Valle

MATERIALS AND METHODS

Yeast Strains and Culture

The *S cerevisiae* strains used in this study are described in TABLE I. Strain DT1100 is isogenic with strain MB1433 except for deletion of that portion of the *pro3* gene which follows codon 83 (codons 84-286). For transformation, the DT1100 strain was grown in minimal medium [0.2 g/100 ml $(NH_4)_2SO_4$, 0.15 g/100 ml yeast nitrogen base without amino acids and $(NH_4)_2SO_4$ (Difco), 2 g/100 ml glucose] supplemented with proline (8.7 mM) and uracil (0.2 mM). Transformants were selected on minimal plates (minimal medium plus 2 g/100 ml Difco agar) supplemented with 8.7 mM proline, replica plated to and scored on minimal plates without proline. Growth rates in minimal medium (supplemented with uracil [0.2 mM] and tryptophan [0.8 mM] for MB1433) were measured in log phase culture.

TABLE I: *S CEREVISIAE* STRAINS USED IN THIS STUDY

Strain	Genotype	Plasmid	Source
MB1433	*MATα trp1 ura3-52*	none	Brandriss [17]
DT1100	*MATα trp1 ura3-52 pro3::TRP1*	none	Brandriss [12]
KD100	*MAT a trp1 ura3-52 pro3::TRP1*	pPRO+.1	this work

cDNA Library

We obtained a human cDNA library, constructed from size-selected (>500 bp) HepG2 (a human hepatoma cell line) cDNA in a yeast expression vector obtained from A. Brake [13]. The promoter and terminator are provided by the *S cerevisiae* glyceraldehyde-3-phosphate dehydrogenase gene. This 2 μM-derived vector contains origins of replication and selectable markers (*URA3* and *ampR*), appropriate for propagation and maintenance in either *S cerevisiae* or *E coli*.

Transformation of Yeast and *E coli*

The entire HepG2 library was used to transform strain DT1100 as described by Schiestl and Gietz [18], with an incubation at 42° C for one hour. Competent *E coli* 294 cells were prepared and transformed as described [19].

Preparation of Plasmid and Insert DNA and Sequencing

DNA was isolated from transformed yeast as described [20] and used to transform 294 cells as above. Plasmid DNA was prepared from *E coli* by standard methods [21]. After digesting the plasmids with Bgl II, the inserts were isolated in low melting point agarose (BRL) and ligated into the BamHI site of pGEM4 (Promega Biotec). Sequencing was performed with the T7 polymerase kit (Pharmacia) or the Sequenase kit (US Biochem) according to the manufacturers' instructions.

Nucleic Acid Analysis

Human genomic DNA was prepared from peripheral lymphocytes as described [22]. Total cellular RNA was isolated from tissue culture cells by guanidium thiocyanate extraction [23]. Poly A+ RNA was isolated by oligo d(T)-cellulose chromatography [24]. RNA was prepared from *S cerevisiae* as by Chapman [25].

DNA transfer and hybridization were as described by Mitchell et al [26]. Reduced stringency hybridizations were performed in a solution of 35% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulfate and 1% (w/v) sodium dodecyl sulfate (SDS). Washes, at reduced stringency, were done twice in 2 x SSC (1 x SSC - 150 mM NaCl, 15 mM sodium citrate), 1% SDS for 10 min at room temperature, twice in 2 x SSC, 1% SDS at 50° C for 30 min and twice in 2 x SSC at room temperature for 30 min. RNA transfer and hybridization were performed using GeneScreen Plus membranes (NEN) following manufacturer's protocols. The probes for these blots were prepared by radiolabelling the appropriate DNA fragments isolated in low melting point agarose using the random hexamer procedure [27]. The huP5CR.1 cDNA probe is the 1.8 kb Bgl II insert of pPRO+.1. A 1.6 kb MB5 mouse tubulin cDNA [28] and a 1.1 kb Bam HI / Hind III fragment of the *S cerevisiae* actin gene [29] were used to control for quality of RNA on the Northern blots. Autoradiograms were quantitated by densitometry using an LKB Ultrascan XL laser densitometer.

Chromosomal Localization

Two human/rodent hybrid cell line mapping panels were used to determine the chromosomal location of the P5C reductase gene. The first, generously provided by T. Mohandas [30], was screened by Southern blotting of Hind III digested DNA and the human P5C reductase cDNA as probe. The second (BIOS Corporation) was screened with the polymerase chain reaction (PCR) using Taq polymerase (Cetus) and primers corresponding to nucleotides 714 - 732 and the complement of nucleotides 1048 - 1066 of the P5C reductase cDNA (FIGURE 3).

Cell Culture

Human cell lines were grown in Eagles' minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum. The cell lines used in this study include skin fibroblasts, human embryonic kidney cells (HEK) (CRL1573), HepG2 cells, MCF7 cells (breast carcinoma cell line), AD12 cells (retinoblasts) (gift of RTMJ Vaessen [31]) and HeLa S3 cells (CCL2.2).

Cell Homogenates and P5C Reductase Assay

Saturated overnight cultures of MB1433, DT1100 or KD100, grown in minimal medium with appropriate supplements, were diluted to an OD_{600} of 0.3 and harvested at an OD_{600} of 0.6. Pellets were resuspended in 0.1 M potassium phosphate buffer, pH 6.8, 1 mM phenylmethylsulfonyl fluoride and the cells were disrupted by vortexing for 30 seconds with glass beads x 4 at 4° C. The beads were washed with additional 0.1 M potassium phosphate buffer pH 6.8 to yield a final homogenate volume of 1 ml. The homogenates were dialyzed overnight in 2 liters of 0.1 M potassium phosphate buffer pH 6.8 at 4° C.

Homogenates of cultured human cells were prepared as described [32]. Protein concentration was determined with the bicinchoninic acid reagent (Pierce) using bovine serum albumin as the standard. The P5C reductase activity was assayed radioisotopically as described [33] except the final concentration of NADH was 2.1 mM.

Reagents

All chemicals not otherwise specified were obtained from Sigma. DL-P5C was made from a commercially available 2,4 dinitrophenylhydrazine derivative of P5C as described [34] with an additional purification step on an ion exchange column [35]. [U-^{14}C]P5C, prepared enzymatically from [U-^{14}C]ornithine (NEN), was a gift of J. Phang. Restriction endonucleases and other enzymes for molecular biology were obtained from Boehringer Mannheim and used according to manufacturer's specifications.

RESULTS

To clone human P5C reductase cDNAs by complementation, we required the appropriate mutant strain of *S cerevisiae* and a human cDNA library. The yeast strain, DT1100, with a partial deletion of its P5C reductase gene (*pro3*) is auxotrophic for proline. As the source of the transforming DNA, we used HepG2 cell cDNA library, size selected for cDNAs >500 bp, in a yeast expression vector [13]. Because HepG2 cells have high P5C reductase activity (TABLE II), we expected that they would be an adequate source for the reductase cDNA. Transformants were selected on a uracil-free minimal medium supplemented with proline. Approximately 10^5 of these were replica plated onto a minimal medium to screen for proline prototrophs. Two colonies, able to grow in the absence of proline, were identified.

We analyzed these two colonies to determine if the Pro+ phenotype was conferred by sequences on the plasmid. To test for cosegregation of the Ura+ and Pro+ phenotypes, we grew the transformants in a medium permissive for plasmid loss (minimal plus uracil and proline). All Ura- colonies were also Pro-, indicating that the sequence complementing proline auxotrophy was located on the plasmid. To characterize the plasmid inserts, we shuttled the recombinant plasmids from the yeast transformants into *E coli*. Both recombinant plasmids (pPRO+.1 and pPRO+.2) had 1.8 kb inserts that hybridized to one another (data not shown). Reintroduction of pPRO+.1 and pPRO+.2 into DT1100 conferred proline prototrophy, confirming that these plasmids carried *pro3*-complementing activity (FIGURE 1). We designated the pPRO+.1 transformed DT1100 strain as KD100.

Figure 1. Growth of *S cerevisiae* strains MB1433, DT1100, and KD100 on a proline-free medium supplemented with uracil and tryptophan for four days at 30° C. The position of the strains is indicated.

To be certain that the complementing activity in KD100 was due to P5C reductase, we measured the activity of the enzyme in extracts of DT1100 and KD100. Extracts of DT1100 had no detectable P5C reductase activity. Extracts of KD100 had low but measurable P5C reductase activity (TABLE II) which was approximately 10% that of normal human fibroblasts and 1-3% that of the proline-prototrophic parental strain (MB1433). To confirm that the relatively small amount of product measured in these assays was produced by an enzyme catalyzed reaction, we showed that it increased linearly over time and coeluted from an ion exchange chromatography column with bona fide radioactive proline (data not shown). We conclude that there is P5C reductase activity in the KD100 yeast albeit at levels much lower than those of MB1433. To determine if this low level of P5C reductase activity was sufficient for normal growth, we compared the growth rates of MB1433 and KD100 in minimal medium. The doubling time for both strains was 2.25 h. The DT1100 strain showed no growth under these conditions.

TABLE II: P5C REDUCTASE ACTIVITY IN *S CEREVISIAE* STRAINS AND HUMAN CELL LINES

S cerevisiae strain	Specific Activity* (nmol proline/hr/mg protein)
MB1433 (*PRO3*)	3010 (2251 - 3548)
DT1100 (*pro3Δ*)	not detectable
KD100 (*pro3Δ* + pPRO+.1)	63 (22 - 109)
Human cell lines	
fibroblasts - control 1	1218 (1187 - 1241)
fibroblasts - control 2	515 (494 - 533)
HepG2	5143 (4929 - 5357)

* mean and (range) of duplicate determinations for each sample. For the yeast samples, two different preparations were assayed.

The inserts from pPRO+.1 and pPRO+.2 were subcloned into pGEM4, resulting in recombinant plasmids designated phuP5CR.1 and phuP5CR.2. The phuP5CR.1 insert was sequenced in its entirety in both directions (FIGURE 2). The nucleotide sequence of the insert and the derived amino acid sequence are shown in FIGURE 3. We sequenced the termini of the phuP5CR.2 insert. The 3' end was identical to that of phuP5CR.1 while the 5' end had five additional base pairs (FIGURE 3), indicating that pPRO+.1 and pPRO+.2 were two independent isolates. The 1805 bp huP5CR.1 cDNA has an open reading frame of 957 bp if the first AUG is the translational start codon. Use of this AUG preserves regions of amino acid identity with the P5C reductases from other species (FIGURE 4). The 957 bp open reading frame encodes a protein of 319 amino acids with a predicted molecular mass of 33.4 kD. The predicted human reductase amino acid sequence is 32% identical to *S cerevisiae*, 38% to *E coli* and *P aeruginosa* and 44% to soybean.

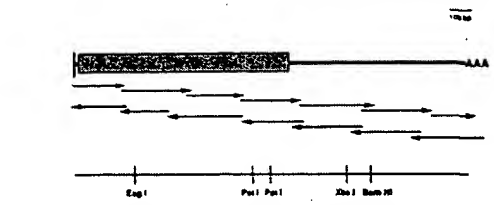

Figure 2. The huP5CR.1 cDNA. The open rectangle denotes the 957 bp open reading frame. The arrows below indicate the sequencing strategy. A simple restriction map is also given. There are no sites for the enzymes Eco RI, Hind III and Rsa I.

FIGURE 5a shows Northern blot analysis of RNA from HepG2 cells, human fibroblasts and various yeast strains probed with huP5CR.1 cDNA. In all human cell line RNA samples, the major hybridizing transcript is 1.85 kb, correlating well with the size of the phuP5CR inserts. Similar results were obtained with RNA from other human cell lines including HeLa, HEK, AD12 and MCF7 (data not shown). The total cellular RNA from both HepG2 cells and fibroblasts have additional minor hybridizing transcripts of 2.25 kb and 1.6 kb. The latter is not present in HepG2 poly A+ RNA. The major hybridizing mRNA in KD100 is 2.0 kb. The slightly larger size of this transcript as compared to that in human cells is due to incorporation of vector glyceraldehyde-3-phosphate dehydrogenase sequences. The intensity of the major hybridizing band in KD100 is ~ 15-fold that in HepG2 cells and ~ 30-fold that in human fibroblasts. As expected, there was no detectable hybridizing transcript in MB1433 or DT1100 despite the presence of approximately similar quantity and quality of RNA in each lane as shown by probing the blot with yeast actin (FIGURE 5b).

Comparison of the amounts of P5C reductase mRNA (FIGURE 5) and enzymatic activity (TABLE II) measured in KD100 extracts shows that the low level of reductase activity in this strain is not due to a low level of the reductase transcript. Rather the low enzymatic activity must be due either to inefficient translation or to post-translational causes. The differences in P5C reductase activity between HepG2 cells and fibroblasts (5 to 10-fold) is partially accounted for by the greater levels of P5C reductase mRNA in HepG2 cells (2 to 3-fold).

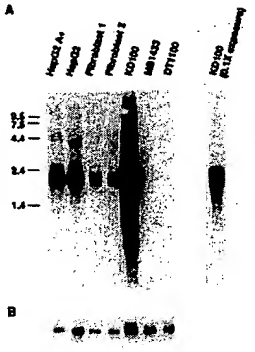

Figure 5. An RNA blot of samples from various human and yeast cells. Panel A: Total cellular RNA from HepG2 cells (10 µg) or cultured fibroblasts (10 µg) or the indicated strains of *S cerevisiae* (20 µg) or HepG2 poly A+ RNA (1 µg) was loaded in the indicated lanes. The blot was hybridized with radiolabelled huP5CR.1 cDNA and the autoradiogram exposed for 48 hours. A one-tenth exposure of the KD100 lane is shown on the right. Panel B: The same blot probed with mouse ß-tubulin (human lanes) or *S cerevisiae* actin (yeast lanes). Standard size markers (kb) are noted.

We analyzed endonuclease digested human genomic DNA to determine the complexity of the fragments detected with the P5C reductase cDNA (FIGURE 6). One or two major hybridizing fragments were present in digests with five different restriction endonucleases, suggesting a single P5C reductase gene with a relatively simple organization. Hybridizing this blot under less stringent conditions (see METHODS) did not reveal any new fragments (data not shown). Using two human/rodent hybrid cell mapping panels, we localized the P5C reductase gene to human chromosome 17 (data not shown).

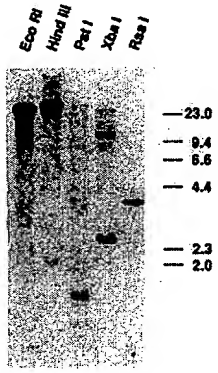

Figure 6. A Southern blot of human genomic DNA hybridized with radiolabelled huP5CR.1. Human genomic DNA (10 µg) was digested with the indicated restriction enzymes. Standard size markers (kb) are noted.

ERRATA

Purification and Properties of Pentachlorophenol Hydroxylase, a Flavoprotein from *Flavobacterium* sp. Strain ATCC 39723

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Volume 173, no. 14, p. 4447, column 2, lines 24 and 25: "μmol" should read "nmol."

Proline Biosynthesis in *Saccharomyces cerevisiae*: Analysis of the *PRO3* Gene, Which Encodes Δ^1-Pyrroline-5-Carboxylate Reductase

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Volume 174, no. 11, p. 3784: Figure 1 should appear as shown below.

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-316                CAGCTG GTACGGAAGC CAACGCAGTC TCCTTTGACA CAACATTTTC TCATTTTAAA GAAGTAGAGG ACTTCCTACA TTTTATTGTC GCTGAGTACA

                              ***** **
-220  TCCAGCAAAA GAAGGTGTAA TATCCTGAGT CACTCCTTAA ACCTACATAC ATTGCCATAG AATGCCATTT ATTACTATAT AAAGTCGCAT ACGTACAAAA GGACAAGATC

                                                                  .  .        .  .  .  .  .  .
-110  TTATCTTCGA AAACAAGAAT ATGAAGGATT TAATAACATT GCCAGGTTAA GAACGGTGTA ACAACAATAG AAGTAACACA ACACCAATAG CAAACAAACC AACTACAGAT

  1   ATG ACT TAC ACA TTG GCA ATT TTA GGC TGC GGT GTT ATG GGC CAA GCA CTT CTT TCC GCC ATT TAT AAT GCT CCA AAG GCG GCT GAT GAA
      met thr tyr thr leu ala ile leu gly cys gly val met gly gln ala leu leu ser ala ile tyr asn ala pro lys ala ala asp glu
                      5                  10                  15                  20                  25                  30

 91   ACT GCT GCT GCA TTT TAC CCT TCC AAA ATT ATC ACA TGT AAC CAT GAT GAA CCT AGT GCA CAA CAA GTT ACC GAT CTA GTT GAG ACA TTC
      thr ala ala ala phe tyr pro ser lys ile ile thr cys asn his asp glu pro ser ala gln gln val thr asp leu val glu thr phe
                      35                  40                  45                  50                  55                  60

181   GAC GAA TCT CCT AAC GGT ATT AAA GTC GAA AGC ACT TAC GGT CAC AAC GTG AGC GCT GTC GAA GAA GCT TCT GTA GTT CTT CTT GGT ACC
      asp glu ser pro asn gly ile lys val glu ser thr tyr gly his asn val ser ala val glu glu ala ser val val leu leu gly thr
                      65                  70                  75                  80                  85                  90

271   AAG CCA TTT TTG GCC GAA GAA GTG TTG AAT GGT GTC AAG AGC GTC ATT GGG GGA AAG CTA CTT ATT TCC CTG GCT GCT GGC TGG ACA ATT
      lys pro phe leu ala glu glu val leu asn gly val lys ser val ile gly gly lys leu leu ile ser leu ala ala gly trp thr ile
                      95                 100                 105                 110                 115                 120

361   GAC CAA TTG AGT CAA TAC ACT AGC ACT GTT TGC CGT GTT ATG ACG AAC ACA CCT GCC AAG TAC GGA TAT GGT TGT GCG GTG GTG TCC TAC
      asp gln leu ser gln tyr thr ser thr val cys arg val met thr asn thr pro ala lys tyr gly tyr gly cys ala val val ser tyr
                     125                 130                 135                 140                 145                 150

451   TCA GCT GAT GTT TCC AAA GAG CAA AAG CCA CTG GTC AAC GAA TTG ATT AGC CAA GTT GGT AAA TAC GTT GAG CTT CCA GAA AAG AAC ATG
      ser ala asp val ser lys glu gln lys pro leu val asn glu leu ile ser gln val gly lys tyr val glu leu pro glu lys asn met
                     155                 160                 165                 170                 175                 180

541   GAT GCT GCT ACG GCT TTA GTC GGT TCA GGC CCC GCT TTT GTT CTC TTG ATG TTA GAA TCC TTG ATG GAG AGT GGG TTG AAA TTG GGA ATC
      asp ala ala thr ala leu val gly ser gly pro ala phe val leu leu met leu glu ser leu met glu ser gly leu lys leu gly ile
                     185                 190                 195                 200                 205                 210

631   CCA TTA CAA GAG AGT AAG GAG TGT GCC ATG AAA GTT CTA GAA GGA ACA GTG AAG ATG GTT GAG AAA AGC GGT GCT CAT CCA TCC GTT TTA
      pro leu gln glu ser lys glu cys ala met lys val leu glu gly thr val lys met val glu lys ser gly ala his pro ser val leu
                     215                 220                 225                 230                 235                 240

721   AAG CAT CAA GTT TGC ACA CCA GGT GGT ACA ACT ATT GCC GGG TTG TGC GTA ATG GAA GAA AAG GGC GTC AAG AGC GGT ATT ATC AAT GGT
      lys his gln val cys thr pro gly gly thr thr ile ala gly leu cys val met glu glu lys gly val lys ser gly ile ile asn gly
                     245                 250                 255                 260                 265                 270

811   GTT GAA GAG GCA GCC CGT GTT GCG TCA CAA TTA GGC CAA AAG AAG AAA
      val glu glu ala ala arg val ala ser gln leu gly gln lys lys lys
                     275                 280                 285

859         TA GATTGCTTTG CGTGAAATGT ATGTATGTTC GCTTTGTAGC CGTATATTAT TTTTTTATTA CTGACCTTGC ATTTTTTCGT CATTTTCCGT CGGTCC
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Molecular Cloning and Evidence for Osmoregulation of the Δ^1-Pyrroline-5-Carboxylate Reductase (*proC*) Gene in Pea (*Pisum sativum* L.)[1,2]

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ABSTRACT

Several cDNA clones encoding Δ^1-pyrroline-5-carboxylate reductase (P5CR, L-proline:NAD[P]$^+$ 5-oxidoreductase, EC 1.5.1.2), which catalyzes the terminal step in proline biosynthesis, were isolated from a pea leaf library screened with a ^{32}P-labeled *Aval* fragment of a soybean nodule P5CR cDNA (A.J. Delauney, D.P.S. Verma [1990] Mol Gen Genet 221: 299–305). DNA sequence analysis of one full-length 1.3-kb clone (pPPS3) indicated that the pea P5CR gene contains a single major open reading frame encoding a polypeptide of 28,242 Da. Genomic analysis suggested that two to three copies of the P5CR gene are present per haploid genome in pea. The primary structure of pea P5CR is 85% identical with that of soybean and exhibits significant homology to human, yeast, and *Escherichia coli* P5CR. The sequence of one of four highly conserved domains found in all prokaryotic and eukaryotic P5CRs is similar to the consensus sequence for the NAD(P)H-binding site of other enzymes. The pea P5CR cDNA hybridized to two transcripts, 1.3 and 1.1 kb in size, in polyadenylated RNA purified from leaf tissues of mature, light-grown plants (4 weeks old). Only the 1.3-kb transcript was detected in younger (1 week old) greened seedlings or in etiolated seedlings. In greened seedlings, steady-state levels of this 1.3-kb mRNA increased approximately 5-fold in root tissues within 6 h after plants were irrigated with 0.4 M NaCl, suggesting that expression of the P5CR gene is osmoregulated.

In a variety of monocot and dicot plant species, adaptation to an environmental stress is accompanied by the accumulation of amino acids and their derivatives (33). One of the best studied responses of this type involves the accumulation of proline under water or salt stress (22, 31). The accumulation of this amino acid results from an increased flux of glutamate to P5C[4] (2) and proline (27) in the proline biosynthetic pathway, as well as decreased rates of proline catabolism (32). In some tissues, proline levels may increase as much as 100-fold in response to stress. In corn roots grown at low water potentials, proline accumulation represented approximately 45% of the total osmotic adjustment (35).

In plants, the regulation of proline synthesis is poorly understood, in part because the proline biosynthetic pathway has not been completely characterized. On the basis of metabolic labeling studies, it appears that stress-induced proline synthesis is the result of a loss of feedback control in the proline pathway at the level of P5C formation (2). This situation is analogous to that in bacteria, in which proline synthesis is known to be regulated at the level of γ-glutamyl kinase via proline feedback inhibition (9). In plants, however, only the last enzyme in the proline pathway, P5CR, has been characterized (7, 19, 21). Evidence for the first two enzymes of this pathway, γ-glutamyl kinase and γ-glutamyl phosphate reductase, is not conclusive (21), and their role in the regulation of proline biosynthesis remains to be demonstrated.

In this context, it is interesting to note that increased P5CR activities and gene expression have been shown in a number of plants in which proline accumulation participates in the process of osmotic adjustment. For example, Triechel (34) found that P5CR activity increased 4-fold in response to NaCl adaptation in the halophyte *Mesembryanthemum nodiflorum*. Similarly, Laliberte and Hellebust (20) reported a 4-fold increase in P5CR activity accompanying salt adaptation in the halophytic alga *Chlorella autotrophica*. Recently, Delauney and Verma (12) cloned a soybean nodule P5CR gene and observed a 6-fold increase in P5CR mRNA levels in the roots of soybean seedlings in response to salinization.

The significance of this apparent osmoregulation of P5CR activity in these plants is unknown, especially because the activity of this enzyme is not modulated in response to osmotic adjustment in other plants (21). There are reports of kinetically distinguishable P5CR isozymes (18) and both cytosolic (18) and chloroplastic (26) forms of P5CR in plants. In view of the possibility that these different P5CRs might play fundamentally different roles in plant amino acid metabolism and stress adaptation, we conducted further studies of this enzyme.

We report here the isolation and characterization of a cDNA clone that encodes a pea leaf P5CR. The P5CR gene was expressed in roots and leaves of both etiolated and light-grown pea seedlings. Increased steady-state levels of P5CR mRNA were observed in the roots of salinized, light-grown seedlings, suggesting that expression of this gene is osmoregulated in these tissues.

[1] The nucleotide sequence for the pea Δ^1-pyrroline-5-carboxylate reductase gene has been deposited in the GenBank/EMBL data bases under accession No. X62842.

[2] Financial support for this research was provided by National Science Foundation grant No. DCB-9117814 to R.D.S.

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[4] Abbreviations: P5C, pyrroline-5-carboxylate; P5CR, pyrroline-5-carboxylate reductase; poly(A), polyadenylation; SSC, standard sodium citrate; pfu, plaque-forming unit; ORF, open reading frame.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli strain PLK-F′ (*recA, hsdR⁻M⁺, mcrA⁻, mcrB⁻, lac⁻, supE, gal⁻*, [*F′, proAB, lacI^Q, lacZΔM15, Tn10*]) was used for cDNA library construction. *E. coli* strain XL1-Blue (*recA1, endA1, gyrA96, thi, hsdR17*($r_k^-m_k^+$), *supE44, relA1, lac⁻*, [*F′, proAB, lacI^Q, lacZΔM15, Tn10*]) was used to plate the cDNA library for screening and for rescue of recombinant pBluescript phagemids from the λZAP II vector (Stratagene, La Jolla, CA).

Construction of the Pea Leaf cDNA Library

Total RNA was extracted from 4-week-old pea (*Pisum sativum* L. cv Wando) leaf tissue using the guanidinium isothiocyanate method, and poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography, as previously described (30). cDNA was synthesized using the ZAP-cDNA kit (Stratagene), according to the manufacturer's instructions. After the poly(A)⁺ RNA (5 μg) was denatured with 10 mM methyl mercury hydroxide, first-strand cDNA was synthesized using Moloney-Murine leukemia virus reverse transcriptase and a poly(T) adapter primer containing an *Xho*I site. After second-strand cDNA synthesis, double-stranded cDNAs were ligated to *Eco*RI adapters, digested with *Eco*RI and *Xho*I, and size fractionated on a Sepharose CL-4B minicolumn.

cDNAs between 400 and 5000 bp in size were collected and ligated to prepared UniZap XR (λZap II) arms. The ligated DNA was packaged in vitro (Gigapak II Gold, Stratagene) and used to transfect *E. coli* PLK-F′ cells. Plaques from 43 individual confluent plates (150 mm) were collected into buffer (1.0 M Tris [pH 7.5], 0.5 M NaCl, 0.15 M $MgSO_4$, 0.01% gelatin). Aliquots from each plate were combined to form a complete library, which contained 1.9×10^6 recombinants.

Isolation of P5CR cDNA Clones

The pea cDNA library was plated with XL1-Blue cells on NZY plates at approximately 2.5×10^4 pfu/plate. Plaques were lifted onto nylon filters (MagnaGraph; Micron Separations, Westboro, MA). Plaque DNA was denatured and UV cross-linked to the filter, which was baked in an 80°C vacuum oven for 1 h. Filters were preincubated in hybridization solution (5× SSPE [0.75 M NaCl, 0.05 M NaH_2PO_4, 6 mM EDTA (pH 7.4)], 2× Denhardt's solution, 50% formamide, 0.1% SDS, 150 μg of salmon sperm DNA) at 40°C for 2 h. Fifty nanograms of a 607-bp *Ava*I fragment of a cDNA encoding soybean nodule P5CR (pProC1, ref. 12) was labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA) to a specific activity of 2 to 5×10^8 dpm/μg using a randomly primed DNA labeling kit (United States Biochemical, Cleveland, OH). The probe was denatured and added to fresh hybridization solution, and the filters were incubated in this solution for 18 h at 40°C. Hybridized filters were washed twice at room temperature with 1× SSC (0.15 M NaCl, 15 mM sodium citrate [pH 7.4]) containing 0.1% SDS and then once at 42°C and exposed to film. Putative P5CR clones were identified on autoradiograms, and the clones were plaque purified. Recombinant pBluescript SK⁻ phagemids were excised in vivo from the λZap II vector by infecting XL1-Blue cells with 200 μL of purified phage (3×10^9 pfu/mL) and 1.2 μL of R408 helper phage (1×10^{11} pfu/ml), according to the manufacturer's instructions (Stratagene). Small-scale preparations of phagemid DNA (16) were used for restriction mapping and sequencing.

DNA Sequencing

Double-stranded phagemid DNA was denatured in 0.2 M NaOH, 0.2 mM EDTA, neutralized by addition of 0.3 M sodium acetate (pH 5), precipitated with ethanol, collected by centrifugation, and dissolved in H_2O. The DNA (3 μg) was sequenced using a Sequenase version 2.0 kit (United States Biochemical). Several restriction fragments of the cDNA insert were subcloned into plasmid pTZ18U to facilitate sequencing. Both strands of the cDNA insert were completely sequenced. Sequence analyses were carried out using the GeneJockey version 1.2 software package (Biosoft, Cambridge, UK) run on a MacIntosh SE computer.

Southern Blot Analysis

Genomic DNA was isolated from pea leaves as described by Richards (28), using the modification of Murray and Thompson (25) in which polysaccharides are removed from the sample by complexation with 1% cetyltrimethylammonium bromide in the presence of 0.7 M NaCl before chloroform extraction and precipitation of DNA. DNA samples were digested with restriction enzymes, and 10 μg of DNA was electrophoresed through an 0.8% agarose gel. The DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 30 min and then by neutralization in 0.5 M Tris (pH 7.5), 1.5 M NaCl. DNA was transferred to a nylon membrane (Magnagraph) by capillary blotting in 10× SSC and fixed to the membrane by UV cross-linking and baking in an 80°C vacuum oven.

The blot was preincubated in hybridization solution (5× SSPE, 5× Denhardt's solution, 50% formamide, 0.5% SDS, 200 μg of salmon sperm DNA) at 42°C for 2 h. A ^{32}P-labeled 830-bp *Eco*RI/*Bam*HI fragment of pea clone pPPS1, encompassing nearly all of the P5CR ORF, was prepared as described above (specific activity of 8.7×10^8 dpm/μg). The probe was denatured and added to fresh hybridization solution, and the blots were incubated for 16 h at 42°C. The blots were then washed twice each with 5× SSC, 0.5% SDS at room temperature and then with 1× SSC, 0.5% SDS and 0.2× SSC, 0.5% SDS, both at 37°C, and exposed to film.

Northern Blot Analysis

Large-scale purification of total RNA from 4-week-old green pea leaf tissues and fractionation of poly(A)⁺ RNA and poly(A)⁻ RNA was carried out as previously described (30). Small-scale isolation of total RNA from 1-week-old pea seedlings used in salt-stress studies (see below) was performed using the method of Chomczynski and Sacchi (8). RNA samples were electrophoresed in a 1% agarose-0.6 M formaldehyde gel and transferred to a nylon membrane (Magnagraph) in 10× SSC. ^{32}P-Labeling of the pea P5CR probe and

northern hybridization were carried out as described above for the Southern analysis. Changes in steady-state levels of P5CR mRNA in response to salt stress were quantitated from autoradiograms using a scanning densitometer (Ultroscan XL; Pharmacia LKB Biotechnology, Piscataway, NJ).

Plant Materials

Pea (*Pisum sativum* L. cv Wando) seeds were planted in washed vermiculite moistened with tap water and grown in darkness or in illuminated growth chambers (16 h of light/8 h of dark) at 22°C for 4 d.

Salinization of Pea Seedlings

Seedlings were irrigated with either tap water or 0.4 M NaCl solutions and grown for an additional 3 d. During this treatment period, root and leaf tissues from both control and salt-treated plants were harvested at 6, 24, and 72 h. Harvested tissues were immediately weighed, quick frozen in liquid N_2, and stored at −80°C before proline determinations or protein and RNA extraction. All manipulations of etiolated seedlings were carried out under green safelights.

Determination of Tissue Proline Contents

The proline content of pea tissues was determined according to the method of Bates et al. (1). Briefly, liquid N_2-frozen tissues were homogenized directly in 3% (w/v) sulfosalicylic acid on ice. Homogenates were centrifuged at 14,000*g* for 20 min at 4°C, and the supernatants were transferred to clean tubes for quantitation of proline. Tissue precipitates were saved for protein analyses (see below). Proline in the 3% sulfosalicylic supernatants was derivatized with ninhydrin and quantitated by A_{520} and comparison with a proline standard curve.

Extraction and Quantitation of Tissue Proteins

Proteins in the 3% sulfosalicylic acid precipitates were solubilized in 1.0 N KOH on ice and the potassium sulfosalicylic acid precipitate was removed by brief centrifugation. Aliquots of the soluble tissue proteins were immediately added to SDS-PAGE sample buffer and boiled to denature the proteins. Protein contents in these samples were determined using the method of Bradford (3).

SDS-PAGE Immunoblot Analysis P5CR Protein Levels in Pea Tissues

SDS-PAGE separations of total soluble proteins from pea tissues, electroblotting onto a nitrocellulose membrane, and immunodetection were carried out as described by Slocum et al. (30) using a rabbit polyclonal antibody produced against SDS-PAGE-denatured soybean nodule P5CR protein, which had been overexpressed in *E. coli* (D.P.S. Verma, personal communication).

RESULTS

Characterization of P5CR Clones

Three cDNA clones, of approximately 150,000 screened from a pea leaf cDNA library, hybridized to a ^{32}P-labeled *Ava*I fragment of the soybean P5CR cDNA pProC1 (12). The putative P5CR clones were characterized by restriction mapping, and one clone (pPPS1) was selected for sequencing. pPPS1 was found to be truncated only four amino acids downstream from the presumed N terminus, based on additional sequence data for the 5′ end of the P5CR gene that was later obtained from two full-length clones, pPPS2 and pPPS3.

P5CR clone pPPS3 contained a 1269-bp insert whose nucleotide sequence is shown in Figure 1. This cDNA comprised 37 bp of 5′-untranslated sequence, a single major ORF of 819 bp initiated by an ATG codon and terminated by the stop codon TAA (OCH), and 391 bp of 3′-untranslated sequence followed by a poly(A) tract. The ORF encoded a polypeptide of 273 amino acids with a predicted molecular mass of 28,242 Da. Codon usage in the pea P5CR gene was not significantly different from that reported for other dicot genes (24).

Two putative poly(A) signals, ATTATA and AAGCTT, were located 34 and 27 bp upstream from the poly(A) tract, respectively (Fig. 1). A third poly(A) signal ATAAAT and a GTTTTCTGCT sequence, similar to the GGUUUUCGCU motif believed to play a role in efficient poly(A) of pea *rbcS* mRNA (23), were located 206 and 290 bp upstream from the poly(A) addition site, respectively.

All three P5CR clones were found to differ from each other slightly over a short stretch of sequence at the extreme ends of their 3′-untranslated regions, just before the poly(A) site. The longest 3′ end was found in pPPS3 (Fig. 1). Nucleotides 1206 to 1214 were missing in pPPS2, and nucleotides 1202 to 1214 were deleted in pPPS1.

Comparison of the pea and soybean P5CR amino acid sequences (data not shown) indicates that the pea sequence was 85% identical with the soybean sequence and, if one takes conservative substitutions into account (Fig. 2), exhibited 94% close homology. This is not surprising because both genes are from leguminous species. In contrast, the pea P5CR sequence was only 34% identical with that of the *E. coli* enzyme (13), 27% identical with yeast P5CR (4), and 36% identical with human P5CR (14).

Although overall amino acid sequence homology among the plant P5CR and other prokaryotic and eukaryotic P5CR was low, several domains within these enzymes were highly conserved. In Figure 2, these conserved sequences are compared, along with the corresponding sequences for P5CR from *Pseudomonas aeruginosa* (29) and the archaebacterium *Methanobrevibacter smithii* (15). Within these domains, sequence identity with pea P5CR ranged from 18% for *M. smithii* to 94% for the soybean enzyme.

Southern and Northern Analyses

Southern blot analysis of pea leaf genomic DNA hybridized to a ^{32}P-labeled *Eco*RI/*Bam*HI fragment of clone pPPS1, encompassing nearly all of the P5CR ORF, indicated that there

```
-36                                        AAACAACACCAGCATTTCATCTGTCACGTAACTAAAC

      Met Glu Ile Leu Pro Ile Leu Ser Asp Ser Tyr Thr Leu Gly Phe Ile Gly    17
    1 ATG GAA ATC CTT CCC ATT CTC TCC GAT TCC TAC ACT CTA GGT TTC ATC GGC

      Ala Gly Lys Met Ala Glu Ser Ile Ala Lys Gly Ala Ser Arg Ser Gly Val    34
   53 GCC GGA AAA ATG GCC GAA AGC ATT GCC AAA GGC GCC TCC CGC TCC GGC GTG

      Leu Pro Ser Ser Arg Ile Val Thr Ala His Ser Asn Pro Ser Arg Arg Ala    51
  104 TTA CCC TCT TCC CGC ATC GTA ACC GCT CAC TCT AAC CCC TCT CGT CGA GCC

      Ala Phe Glu Ser Ile Gly Ile Thr Val Leu Ser Ser Asn Asp Asp Val Val    68
  155 GCC TTC GAG TCC ATC GGC ATC ACC GTC CTT TCT TCC AAC GAC GAT GTC GTT

      Arg Ala Ser Asn Val Val Val Phe Ser Val Lys Pro Gln Leu Val Lys Asp    85
  206 CGT GCC AGC AAC GTC GTC GTT TTC TCG GTT AAA CCT CAA TTA GTG AAG GAC

      Val Val Leu Lys Leu Lys Pro Leu Leu Thr Lys Asp Lys Leu Leu Val Ser   102
  257 GTT GTG TTG AAA TTG AAG CCG CTC CTA ACT AAG GAC AAG CTT TTG GTT TCT

      Val Ala Ala Gly Ile Lys Leu Lys Asp Leu Gln Glu Trp Ala Gly His Glu   119
  308 GTT GCT GCT GGA ATC AAG TTG AAA GAT CTT CAG GAA TGG GCT GGC CAC GAA

      Arg Phe Ile Arg Val Met Pro Asn Thr Pro Ala Ala Val Gly Gln Ala Ala   136
  359 AGA TTT ATA AGG GTA ATG CCA AAT ACA CCT GCT GCA GTT GGC CAG GCG GCA

      Ser Val Met Ser Leu Gly Gly Ala Ala Thr Glu Glu Asp Ala Asn Leu Ile   153
  410 TCA GTT ATG AGC TTG GGA GGA GCT GCA ACA GAA GAA GAT GCA AAT CTT ATA

      Ser Gln Leu Phe Gly Ser Ile Gly Lys Ile Trp Lys Ala Asp Asp Lys Phe   170
  461 TCC CAA TTA TTT GGG TCA ATT GGC AAA ATA TGG AAA GCT GAT GAT AAG TTT

      Phe Asp Ala Ile Thr Gly Leu Ser Gly Ser Gly Pro Ala Tyr Ile Tyr Leu   187
  512 TTT GAT GCA ATA ACT GGT CTA AGT GGC AGT GGT CCT GCT TAT ATT TAT TTA

      Ala Ile Glu Ala Leu Ala Asp Gly Gly Val Ala Ala Gly Leu Pro Arg Asp   204
  563 GCA ATA GAG GCT CTG GCT GAT GGA GGA GTA GCA GCT GGT CTA CCG CGT GAT

      Leu Ala Leu Ser Leu Ala Ser Gln Thr Val Leu Gly Ala Ala Ser Met Ala   221
  614 CTT GCA TTA AGT CTA GCT TCT CAG ACT GTA CTA GGA GCA GCA TCA ATG GCA

      Thr Leu Ser Gly Lys His Pro Gly Gln Leu Lys Asp Asp Val Thr Ser Pro   238
  665 ACC CTT AGT GGG AAG CAT CCA GGA CAG CTC AAG GAT GAT GTC ACT TCT CCT

      Gly Gly Thr Thr Ile Ala Gly Val His Glu Leu Glu Lys Gly Gly Phe Arg   255
  716 GGC GGG ACA ACA ATT GCA GGC GTT CAT GAG TTG GAA AAA GGG GGT TTC CGT

      Gly Thr Leu Met Asn Ala Val Val Ala Ala Ala Lys Arg Ser Arg Glu Leu   272
  767 GGA ACA CTG ATG AAT GCT GTT GTT GCT GCC GCT AAA CGC AGC CGA GAG CTT

      Ser Stop                                                              273
  818 TCC TAA ACCTATAGGTGAGGATCCTATAGCAGCAGAGAGCTTTCCTAAATGGGCGAGGATCCTAT
  883 TATTTCTAAGAGAAAACTTTGATTTCAGTAGAGTTTTCTGCTTACATATAGTATGTCAAAGTAGCCT
  950 TCTTTGAGATGAGTAGTAGTTGCGGAAATGTTTAGTCTTAGAAACTTCTTTTATAAATGCTCTACTA
 1017 GTATAGACGATTTCAGCCAAGTAGCTCAGAATAACAGCCAGTCCCGCTTATTGTTGCTTGCCGGGGC
 1084 TTGACGTTTCTTTACTGAAGACGATCATTAAGAACAATTTGGCCACTAGACATGGATTCACATGTAC
 1151 ATGCAGTTATGCATTTTGTCTCTAATTATATCAAGCTTATTTCGTTTTGCCTTTGAAAGGTTCCAAA
 1218 AAAAAAAAAAAAAA
```

Figure 1. Nucleotide sequence of clone pPPS3 and deduced amino acid sequence for pea P5CR. Putative 3'-end poly(A) sequences are indicated by underlining. The numbering system refers to the DNA sequence starting at the first ATG as +1.

are approximately two to three copies of the P5CR gene per haploid genome (Fig. 3). Northern blot analyses of poly(A)$^+$ RNA isolated from leaf tissues of mature greened seedlings showed two distinct transcripts 1.3 and 1.1 kb in size (Fig. 4). The 1.1-kb transcript was not detected in total RNA extracted from leaves or roots of younger (1 week old) greened or etiolated seedlings (Fig. 5), but low levels of this transcript were observed in total RNA from leaves of 3-week-old light-grown seedlings (data not shown).

Effect of Salt Stress on Levels of P5CR mRNA and Protein and Proline in Leaf and Root Tissues

In greened pea seedlings, steady-state levels of the 1.3-kb mRNA in leaf tissues were approximately 5-fold higher than in root tissues in control plants (Fig. 5). Irrigation of these seedlings with 0.4 M NaCl resulted in a 5-fold increase in levels of the 1.3-kb P5CR mRNA in root tissues within 6 h, as compared with control seedlings (Fig. 5). Root P5CR mRNA levels remained 4- and 3-fold higher in salt-stressed plants at 24 and 72 h, respectively. In contrast, there was no significant influence of salinization on levels of the 1.3-kb transcript in leaf tissues of these seedlings.

Similarly, salt treatment had no effect on levels of the 1.3-kb mRNA in either the roots or shoots of etiolated seedlings (Fig. 5). However, in the etiolated seedlings, transcript levels were 5-fold higher in roots than in leaves. The 1.3-kb mRNA also increased approximately 6-fold in roots of control seedlings during the 3-d course of the experiment.

Changes in P5CR protein levels in these seedlings were estimated by immunoblotting using a soybean nodule P5CR antibody, which weakly recognized an M_r 28,000 protein in greened and etiolated tissues. In all tissues, levels of the presumed P5CR protein were poorly correlated with levels of 1.3-kb mRNA in control or salinized seedlings, remaining essentially constant (data not shown).

Proline contents of the pea seedlings are shown in Figure 6. In the leaves and roots of untreated greened seedlings, proline levels reflected 1.3-kb mRNA levels, being approximately 5-fold higher in leaves. In salinized seedlings, proline accumulated to the same extent in both leaves and roots, again reflecting the increase in root 1.3-kb mRNA to levels approximating those of leaf tissues (Fig. 5). Proline levels in salt-stressed roots, in which there was a rapid increase in 1.3-kb mRNA levels, increased 12-fold over controls.

In etiolated seedlings, levels of 1.3-kb mRNA and proline accumulation were poorly correlated. Proline levels in the leaf were 2-fold higher than in roots, despite the fact that 1.3-kb mRNA levels were 5-fold higher in the latter tissues. Salt-induced proline accumulation was approximately equal in leaf and root tissues, although the magnitude of the response was higher in the root.

DISCUSSION

The pea P5CR gene characterized in this study exhibits a single major ORF that encodes a protein containing 273

```
pea                (98-106)  KLLVSVAAG  (122-130)  IRVMPNTPA
soybean            (99-107)  KLLVSVAAG  (123-131)  IRVMPNTPA
human              (88-96)   DRHIVVSCA   (118-126)  IRCMTNTPV
yeast              (109-117) KLLISLAAG   (131-139)  CRVMTNTPA
E. coli            (91-99)   SLVVSIAAG   (116-124)  IRAMPNTPA
P. aeruginosa      (91-99)   QLIVSIAAG   (116-124)  VRCMPNTPA
M. smithii         (90-98)   TQIIHTCAG   (115-123)  ISSTYDEDN

pea                (172-185) DAITGLSGSGPAYI (227-246) HPGQLKDDVTSPGGTTIAGV
soybean            (173-186) DAITGLSGSGPAYV (228-247) HPGQLKDDVTSPGGTTITGI
human              (168-181) DAVTGLSGSGPAYA (223-242) HPGQLKDNVSSPGGATIHAL
yeast              (181-194) DAATALVGSGPAFV (236-255) HPSVLKHQVCTPGGTTIAGL
E. coli            (166-179) HPVVGVSGSSPAYV (221-240) HPGALKDMVCSPGGTTIEAV
P. aeruginosa      (167-180) DAVTAVSGSGPAYF (222-241) EPAELRRRVTSPNGTTEAAI
M. smithii         (166-179) EIATIAASCMPAFI  (221-240) SPDELINKVATKNGITQKGL
```

Figure 2. Conserved sequences among prokaryotic and eukaryotic P5CRs and comparison with the pea P5CR amino acid sequence. Residues with a line above indicate identity with pea residues; conservative substitutions are indicated with two dots over the residue. Conservative substitutions are defined as: (a) C; (b) S, T, P, A, G; (c) N, D, E, Q; (d) H, R, K; (e) M, I, L, V; (f) F, Y, W (10). The underlined pea sequence is similar to the GTGIAP motif occurring within the consensus sequence for the putative NAD(P)H-ribose-binding site of several enzymes (6).

amino acid residues, assuming that the first ATG is the translational start codon (Fig. 1). Several lines of evidence support this assumption. First, use of this codon preserves amino acid sequence homology between the pea and soybean P5CR enzymes. Direct amino acid sequence data for N-terminal (residues 1–15) and internal peptide fragments (residues 221–241) from purified soybean nodule P5CR (7) confirm the translated sequences. Second, the major ORF of the pea P5CR gene encodes a polypeptide with a predicted M_r of 28,242, and the soybean nodule P5CR antibody recognizes an M_r 28,000 polypeptide from pea tissues on SDS-PAGE immunoblots. Finally, the predicted size for the pea enzyme is also similar to that for P5CRs purified from other plant species (7, 19) and from *E. coli* (13).

Unlike P5CRs characterized from other organisms, neither the pea nor soybean enzyme contains any Cys residues.

The functions of the highly conserved domains within P5CR proteins (Fig. 2) are unknown. However, one of these domains, spanning residues 235 to 266 of the pea enzyme, may be involved in binding the NAD(P)H cofactor. Its sequence fits the overall consensus sequence characteristic of the $\beta\alpha\beta$-fold involved in binding the ADP moiety of flavin adenine dinucleotide/NAD cofactors (37). The occurrence of a hydrophobic Ala residue at position 266, in place of the acidic residues Asp or Glu, further suggests that the pea P5CR encoded by clone pPPS3 would utilize NADPH rather than NADH as a cofactor (37). This domain also contains a GTTIAG sequence (Fig. 2), which is similar to the GTGIAP motif found within the NAD(P)H-ribose-binding site of nitric oxide synthase and several other enzymes utilizing this cofactor (6).

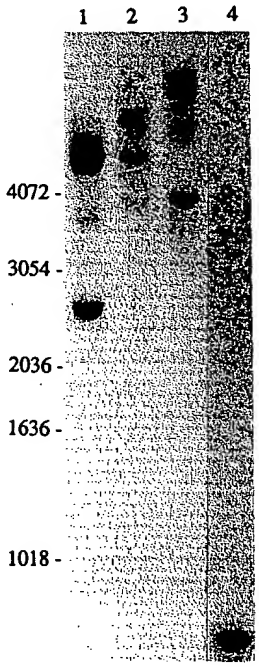

Figure 3. Southern blot analysis of pea leaf genomic DNA hybridized with a ^{32}P-labeled 832-bp *EcoRI/BamHI* fragment of clone pPPS1. DNA (10 μg) was digested with *EcoRI* (lane 1), *KpnI* (lane 2), or *SacI* (lane 3), which do not cut at any sites within the clone. Reconstruction lane 4 contains two gene copy equivalents of probe DNA. DNA size markers (kb) are indicated.

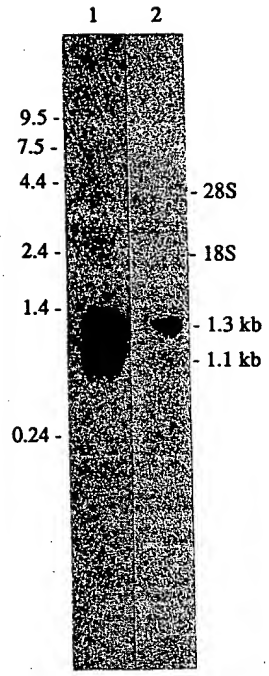

Figure 4. Northern blot analysis of pea leaf poly(A)$^+$ RNA (5 μg; lane 1) and poly(A)$^-$ RNA (10 μg; lane 2) hybridized with a ^{32}P-labeled 832-bp *EcoRI/BamHI* fragment of clone pPPS1. Positions of the 28S and 18S rRNA bands and RNA size markers (kb) are indicated.

To date, all of the P5CR clones that we have characterized appear to encode the same cytosolic enzyme, represented by the single 1.3-kb transcript seen on northern blots. The N-terminal sequence for the pea P5CR polypeptide does not exhibit the general characteristics of presequences associated with proteins imported into mitochondria or chloroplasts, such as an abundance of hydrophobic residues and a net positive charge (36). Soybean nodule (18) and yeast (5) P5CRs have also been reported to be cytosolic proteins. However, chloroplast-localized P5CR activities have been reported for both greened and etiolated pea tissues (26).

Northern analysis of pea leaf poly(A)$^+$ RNA from mature (4 week old) leaf tissue indicates that two distinct P5CR transcripts, 1.3 and 1.1 kb in size, occur in this tissue. In younger pea and soybean (12) seedlings, only the 1.3-kb transcript is expressed. The occurrence of two different transcripts in mature seedlings may result from expression of different P5CR genes, differential splicing of transcripts, or the use of different poly(A) sites during transcript processing. Dougherty et al. (14) speculated that differential splicing of the P5CR transcript in human hepatoma cells, in which there appears to be a single copy of the P5CR gene, might give rise to the kinetically distinguishable P5CR isozymes that have been characterized in different animal tissues.

Multiple poly(A) patterns have been shown for several plant genes (11), and analysis of pea P5CR clone pPPS3 (Fig. 1) suggests that the two pea transcripts may result from the use of different poly(A) signals. Unlike the soybean P5CR

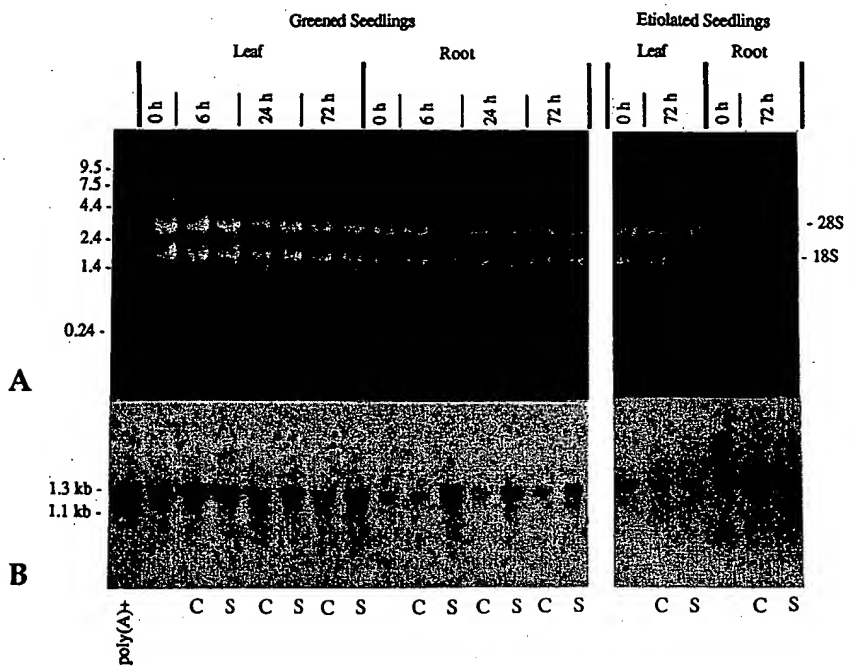

Figure 5. Northern analysis of changes in steady-state levels of the P5CR 1.3-kb transcript in response to salinization in etiolated and greened pea seedlings. Total RNA (20 μg/lane) was isolated from leaf and root tissues 6, 24, or 72 h after irrigation with 0.4 M NaCl. The positions of the 28S and 18S rRNA bands and RNA size markers (kb) are indicated (A). The corresponding northern blot (B), probed with a ^{32}P-labeled *Eco*RI/*Bam*HI fragment of clone pPPS1, shows the 1.3- and 1.1-kb P5CR transcripts in the poly(A)$^+$ RNA lane (5 μg) and the 1.3-kb transcript in control (C) and salt-treated (S) seedlings.

gene, which appears to use the canonical AATAAA poly(A) signal characteristic of animal genes, the pea P5CR gene resembles most other plant genes that have been studied in that it uses poly(A) signals that have diverged significantly from the AATAAA motif (11, 17). The putative poly(A) signals in the pea P5CR gene are clustered at two positions that would be expected to produce transcripts of approximately 1.3 and 1.1 kb in size. Even within the 1.3-kb transcript, however, the actual site of poly(A) differs among the three P5CR clones that we have characterized, occurring at three discrete loci over a span of 13 nucleotides. This may be attributable to the fact that two different putative poly(A) signals are located proximal to one another about 30 bp upstream from the poly(A) tract in the pea P5CR gene.

Steady-state levels of the 1.3-kb transcript increase in the roots of light-grown seedlings upon salt treatment, suggesting that P5CR gene expression is osmoregulated in these tissues. In contrast to the previous report that salt-inducible P5CR expression occurred in the roots of etiolated soybean seedlings (12), we found no evidence for this in either roots or leaves of etiolated pea seedlings. The reason for this discrepancy is unknown, but the results of our studies suggest that patterns of P5CR expression are influenced by light. Tissue-specific differences in P5CR expression may reflect fundamental changes in the dynamics of proline metabolism in root and leaf tissues in response to light. Furthermore, the appearance of the 1.1-kb mRNA only in older light-grown seedlings suggests that the expression of this P5CR transcript may be developmentally regulated in light-grown plants.

The significance of the observed salt-inducible expression of the P5CR gene, with regard to a possible role for P5CR in the regulation of proline synthesis, is unclear. Changes in P5CR transcript levels, in response to salinization, are not seen in etiolated seedlings or in leaves of greened seedlings, although proline accumulation is observed in all salt-stressed tissues. In the roots of greened seedlings, increased transcript levels do approximately parallel a marked increase in proline levels. In all tissues, however, P5CR protein levels appear to remain more or less constant, irrespective of changes in 1.3-kb mRNA levels. It is possible that salt stress simply induces the synthesis and/or stabilization of P5CR mRNA without leading to increased synthesis or accumulation of P5CR protein. We did not measure P5CR activities in the present study, but if one assumes that activity levels reflect P5CR protein levels in the pea tissues, then it seems reasonable to conclude that proline accumulation in the salt-stressed seedlings is not modulated by changes in P5CR activity but is regulated at the level of P5C formation, as has been suggested by previous workers.

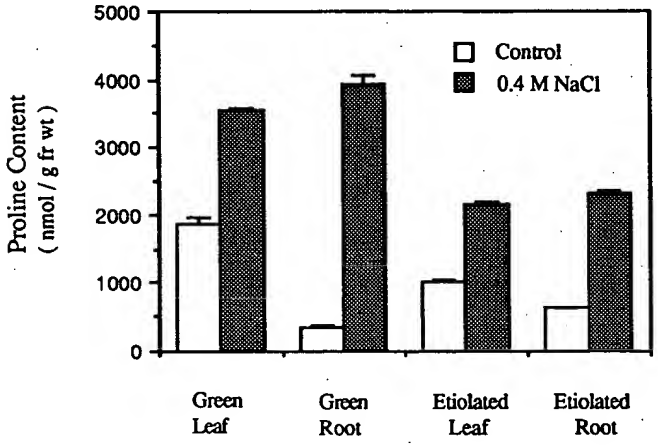

Figure 6. Proline contents of leaf and root tissues of pea seedlings 72 h after irrigation with 0.4 M NaCl. Data represent three replicates. SE bars are indicated. fr wt, Fresh weight.

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